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**The Molecular Weight Distributions of Bacterial
Cellulose as a Function of Synthesis Time**

Gerard J. F. Ring

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THE MOLECULAR WEIGHT DISTRIBUTIONS OF BACTERIAL CELLULOSE
AS A FUNCTION OF SYNTHESIS TIME

A thesis submitted by

Gerard J. F. Ring

B. S. 1974, State University of New York at Albany

M.S. 1976, Lawrence University

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Appleton, Wisconsin

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"I learned that in mathematics one depends on inspiration,
in science on experimental evidence,..."

G. W. Leibniz

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SUMMARY

The kinetics of the biosynthesis of isolatable bacterial cellulose produced by the organism Acetobacter xylinum was investigated by monitoring the yield, the number-average molecular weight, and the weight-average molecular weight as functions of synthesis duration. The simultaneous measurement of all three parameters made it possible to observe the increase in the number of polymers as well as their relative degree of elongation at any given time. The experimental objective of this study was to determine whether or not cellulose polymers are stochastically polymerized from monomeric or polymeric cellulose precursors that are not directly connected to a synthesizing enzyme. A theoretical Poisson polymerization function was utilized as an appropriate model to calculate, as functions of time and precursor molecular weight, the yield and the average molecular weights to be expected from a random polymerization. Theoretical results were then compared with equivalent experimental results.

The molecular weight averages were obtained from the gel permeation chromatography (GPC) chromatograms of the bacterial cellulose tricarbanilate derivatives. To ensure accurate molecular weight determination, a dispersionally correct universal calibration was developed to account for the large differences between the instrumental spreading coefficients for the polystyrene calibration standards and the tricarbanilates. Because bacterial cellulose normally resists derivatization, it was also necessary to utilize an accessibility procedure prior to derivatization in order to open its microfibrillar structure for complete tricarbanilation. The procedure entailed initial preparation of a methylol derivative, regeneration in warm sodium sulfite, repeated washings with water, and freeze-drying to yield the substantially modified but undegraded cellulose.

The experimental results demonstrated that the rate of increase in the number of cellulose molecules, the rate of cellulose-mass accumulation, and the relative rate of cellulose-chain elongation are described by approximate first-order kinetics

characteristic of bacteria in their logarithmic growth phase. A Poisson polymerization function predicts that these rates will also be first-order, but the interrelationship between the rate constants for each experimental variable indicated that a stochastic mechanism does not occur. For a random process, the three rate constants should be approximately equal; however, the experimentally determined constants were not. The values for the rate constants increased in the order of their listing, with the cellulose chain elongation rate constant being the largest. The substantially larger experimental rate constant found for the relative rate of chain elongation were interpreted to be a result of the cellulose chains being constantly connected to only one synthesizing enzyme. Each polymerization enzyme then conceivably serves to elongate just one cellulose chain rather than several. It was also demonstrated that a theoretical, random mechanism would predict polydispersities never greater than a value of 2; experimental values much greater than this were actually obtained.

INTRODUCTION

The importance of cellulose as a structural material in the plant kingdom is easily recognized by the fact that it is the most abundant organic material known (1). Naturally occurring cellulose is rapidly polymerized from nucleoside diphosphoglucose into a mechanically strong, yet flexible, matrix. Depending upon the source, it is also associated to varying degrees with other noncellulosic components that necessitate purification by chemical extraction. Consequently, the elucidation of the structure and formation of cellulose has been hampered by the consideration that all known isolation techniques alter to some extent its natural form and may even degrade the polymers (2). Thus, any purified natural cellulose is reliant on its isolation procedure as an integral part of its description.

A study of the kinetics of cellulose biosynthesis is particularly vulnerable to isolation techniques which will remove low molecular weight polymers that have not yet been consolidated into microfibrillar structures. The solubilization of this material will have the detrimental effect of reducing the total mass and increasing the apparent number-average degree of polymerization. But, at the present state of the art, it is impossible to avoid solubilizing low molecular weight material (3). Therefore, a study of cellulose polymerization kinetics will not be representative of the total production of cellulose, only the production of isolatable cellulose.

Kinetic studies involving the remarkably pure extracellular cellulose produced by the bacterium Acetobacter xylinum have demonstrated that the yield of isolatable bacterial cellulose as a function of time follows the same rate of increase as the culture's total mass accumulation (4,5). This observation has been interpreted as indicative of an essentially complete cellulose isolation. Furthermore, the relatively mild isolation procedures that were utilized have been demonstrated to be non-degradative (6). Therefore, bacterial cellulose represents an almost ideal source

where the molecular weight distribution and the molecular weight averages of cellulose have undergone a minimum of modifications.

The significance of being able to accurately measure the molecular weight averages with time as more and longer molecules are produced is that the mechanisms of polymerization can be elucidated if the proper types of averages are measured. The previous investigations, however, have only been able to obtain the weight-average molecular weight and not the number-average molecular weight, which is necessary to estimate the actual increase in the number of polymers. Therefore, those studies could not prove with certainty a particular mechanism for cellulose production.

Accurate number-average molecular weights are difficult to obtain because the high molecular weights attained by bacterial cellulose are generally above the sensitivity limits of membrane osmometers. Furthermore, the relatively short synthetic intervals involved preclude obtaining sufficient material for more than one absolute measurement; hence, a weight average is usually preferred. In this study, however, these drawbacks were eliminated by the utilization of gel permeation chromatography (GPC). The technique of GPC yields both number-average and weight-average molecular weights simultaneously without recourse to duplicate samples. But, because it is a relative technique rather than an absolute technique, the measurement of accurate molecular weight averages relies on the determination of an accurate calibration. An accurate calibration depends on the proper assignment of molecular weight averages to their proper positional values on the calibration curve. When an accurate calibration is obtained, the molecular weight averages calculated from every GPC chromatogram must then be corrected for the effects of instrumental spreading or peak dispersion, which tends to raise the value of the weight-average and lower the value of the number-average molecular weight.

When there is difficulty in obtaining calibration standards for the type of polymer being studied, it is customary to utilize an alternative series of calibration standards and translate the calibration so obtained via the Mark-Houwink constants for both polymers into the calibration desired. This is known as a universal calibration. Peak dispersion is also a factor when universal calibration is used, but the dispersion correction must then compensate for two different polymers. Until recently, however, dispersion effects for universal calibration have been ignored. But, in order to achieve a higher degree of accuracy for this study, it was found necessary to derive a dispersionally correct universal calibration. A detailed presentation of the development of this new universal calibration appears in Appendix II.

The primary objective of this study was to determine the existence or nonexistence of unconsolidated, soluble, polymeric cellulose precursors as proposed by Colvin and Leppard (7). Because a polymeric precursor would have a distinctly more discrete molecular weight distribution, it was realized that the effects of a polymeric precursor could be detected by observing the change in the molecular weight distribution with synthesis time. This would also mean that the molecular weight averages would vary in a different manner as opposed to a monomeric polymerization. Thus, GPC was utilized as the experimental method to obtain the molecular weight distributions and their molecular weight averages. A Poisson polymerization function was selected to determine the theoretical variations in the molecular weight averages due to precursor molecular weight because it ideally modeled the hypothesized property of soluble precursors randomly attaching to any previously consolidated cellulose chain, and it has also been previously proposed for bacterial cellulose biosynthesis (4).

BACTERIAL CELLULOSE PRODUCTION

DESCRIPTION OF ACETOBACTER XYLINUM

The genus Acetobacter is a group of gram-negative bacteria with the ability to oxidize ethanol to acetic acid. In particular, the bacterium Acetobacter xylinum, noted for its ability to produce vinegar, is usually found in wine vats as "...a sort of moist skin, swollen, gelatinous and slippery,..." (8). It has been referred to as "mother-of-vinegar" and was the basis of the early vinegar industry. The gelatinous skin, a polysaccharide matrix within which the bacterial cells are enmeshed, is more commonly known as a pellicle.

Because Acetobacter xylinum is an obligate aerobe requiring a constant supply of oxygen, the function of the polysaccharide pellicle may be to provide a buoyant environment at the air-liquid interface. This idea finds some support in the observation of "pellicle-balloons" filled with carbon dioxide in constantly agitated cultures. However, the pellicles of A. xylinum are commonly found in a membranelike condition in nonagitated cultures. The polysaccharide of the pellicle is predominantly cellulose derived biochemically from various possible sugar analogs (9), and its biosynthesis has been the subject of numerous investigations (10).

MICROFIBRIL FORMATION

Bacterial cellulose has the same native crystal structure as cotton cellulose (11). In contrast to cellulose from green plants, however, it is not deposited into multilayered walls surrounding the cell. Rather, it is formed into numerous ribbons, each consisting of several microfibrils, interlaced into a matted pellicle structure (12). The individual ribbons are of various lengths and are subject to continued elongation from the addition of glucose monomers (13). It is known that live, biosynthetically active bacterial cells are responsible for this elongation

process (4); however, the exact role which the cells play in this process is still controversial (3). The primary polymerization of glucose into polyglucosan chains (β -1,4-polyanhydroglucose) is believed to be performed by a synthetase enzyme complex, situated within the bacterial cell envelope (14), which extrudes these chains extracellularly through pores in the surrounding outer membranes (15,7,16). But the manner in which these chains are consolidated into microfibrils, and then microfibrillar ribbons, has not as yet been agreed upon.

One proposal (15,16), based on evidence from microscopy postulates that the polymerization enzymes are arranged in a linear array that allows the connected polymer chains to immediately crystallize into about 46 continuous microfibrils, and that these microfibrils are then consolidated into a single, unified ribbon extruding from the polar region of the cell. During cellular division, this linear array is bisected such that the mother and daughter cells will each receive half of the synthetase sites, but with no curtailment in the activity of these sites (50). Initially, the newly divided cells will produce cellulose ribbons of a reduced width until the biosynthesis of new enzyme complexes restores their original number per cell. Thus, as each new complex is activated, the incorporation of new microfibrils will increase the width of the already-established, microfibrillar ribbon. In this manner, the process of cellular replication does not interfere with the ongoing process of cellulose biosynthesis.

In contrast to this proposal, it has been suggested that the synthetase enzymes are not attached to the growing cellulose microfibrils, but, instead, produce oligomeric precursors that are excreted to the external milieu (7). These precursors then form nascent, highly hydrated fibrils via a presumably simple but random polymeric association process, followed by an end-to-end covalent bonding of the precursors to form substantially longer molecules after they have associated into nascent microfibrils. Finally, this second proposal also assumes that the initiation of new

microfibrils is a random process, which occurs from the chance association of several precursors to form an "acceptor," identical to the growing tip of a previously formed microfibril.

BACTERIAL CELLULOSE KINETICS

When bacterial cellulose is harvested by extracting the noncellulosic components with NaOH under nitrogen, the yield of the isolated cellulose increases with time at the same rate as the total dry mass (5,4). This rate is described by an exponential function that is typical of the logarithmic growth phase of bacterial cultures (17). This function, known as the law of bacterial growth, explicitly describes the total mass M of a culture after time t and has the form:

$$M = M_0 (k_{ic} + k_{as}) e^{\alpha t}, \quad (1)$$

where M_0 is the initial mass at $t = 0$, α is the first-order rate constant, and k_{ic} and k_{as} are the weight fractions for the isolated cellulose and the alkali-soluble material, respectively. Theoretically, during the logarithmic growth phase, the bacterial cells are experiencing maximum enzymatic activity for the prevailing reaction conditions due to a nonlimiting supply of nutrients and oxygen. These investigations (4,5) have also demonstrated that the increase in the number of cells conforms to the law of bacterial growth with approximately the same rate constant α as the rate of mass accumulation [within 3% for values of $\alpha = 0.08$ to 0.15 (4)],

$$N = N_0 e^{\alpha t}, \quad (2)$$

where N_0 is the initial number of cells at time $t = 0$, and N is the total number after time t .

It has been shown experimentally that the ratio k_{ic}/k_{as} may range from a value of 0.65 to a value of 1.33 for various cultures (4); however, it is also known that

alkali extraction will remove a polyglucosan of relatively low molecular weight (13,18,19). Therefore, it is possible to assume that the parameter k_{1c} does not represent the entire cellulose polymerization production, and that k_{as} represents some soluble cellulose as well as noncellulose material. But, because the isolated cellulose does follow the law of bacterial growth, the mass of isolated cellulose is proportional to the total cellulose polymerization product,

$$w = w_0 (k_i + k_s) e^{\alpha t}, \quad (3)$$

where w_0 and w are the initial and final masses, respectively, of the total cellulose polymerization product; k_i is the weight-fraction of the isolatable cellulose; and k_s is the weight-fraction of the soluble, or nonisolatable cellulose. Thus, the observation that the yield of isolated cellulose follows the law of bacterial growth is very significant, because it demonstrates the alkali-extracted bacterial cellulose can be considered as a distinct enzymatic product regardless of whether or not it represents the total cellulose polymerization product.

With first-order kinetics, an average period during which the number of cells will double, τ_{cell} , can be calculated from the first-order rate constant α :

$$\tau_{cell} = \ln 2 / \alpha. \quad (4)$$

This average time is interpreted as the time necessary for a typical cell to reproduce and is commonly referred to as the generation time. During each generation the appearance of new cells is accompanied by the production of new cellulose molecules. However, it is also possible that at the time of cellular replication the polymerization of older molecules may be terminated. If this were true, then the measurable average length or degree of polymerization of the cellulose molecules experiencing such a termination would rapidly reach a constant value. But it has been observed that Acetobacter xylinum does not produce a constant degree of polymerization.

Rather, the DP slowly increases over a period of several generations (4,20). Thus, the evidence for A. xylinum suggests that no such termination reaction is present and that the cellulose molecules, once initiated, continue to grow for the life of the cells. For this reason, it has been proposed that a possible mechanism for bacterial cellulose polymerization is a stochastic "living-polymer" process described by a Poisson function (4).

THEORETICAL AND EXPERIMENTAL OBJECTIVES

A Poisson probability function can be defined as a mathematical expression which predicts the likelihood that a certain number of random events will occur during any particular time period, given that the occurrence of each separate event is independent of the occurrence of the other events, and that it is impossible for two events to occur simultaneously at the same position (21). As a consequence, the Poisson function is discrete, disallowing fractional events.

The utilization of a Poisson function as a predictor of the chain-lengths of isolated bacterial cellulose requires that an "event" be defined in terms of the degree of polymerization. Specifically, a Poisson function implies that, during synthesis, each molecule of cellulose which is incorporated into the mass of isolated cellulose may experience a random number of events, whereby its polymeric length would be stochastically increased by subunits of equal size or molecular weight (22). If an event is defined in terms of the incremental change in DP, then the magnitude of this incremental change becomes important, particularly in light of the controversy that exists about the mode of microfibril formation. For the case of simultaneous polymerization and crystallization, an event would correspond to the polymerization of cellulose by a subunit of glucose or perhaps cellobiose (23). However, for the case of oligomeric precursors, an event would correspond to the bonding of an oligomer either onto another oligomer, or a cellulose chain composed of repeated oligomers. This then postulates that if a Poisson function is applicable to the formation of isolated bacterial cellulose, each polymer would have a length that could be measured in multiples of the subunit size or degree of polymerization.

Because of the important implications regarding the mechanisms of microfibril formation, it is the purpose of this investigation to document the simultaneous

rates of mass accumulation and DP attainment by measuring the cellulose yield, number-average and weight-average degrees of polymerization as functions of time, and to compare these experimental values against the theoretically calculated values for a Poisson regulated growth-pattern. This requires deriving explicit expressions for the experimental parameters in terms of the Poisson function. It also requires defining the independent variables for each expression, including the molecular weight of the event subunit, in such a manner that they can be measured unambiguously.

THE POISSON POLYMERIZATION FUNCTION

The process of polymer introduction is of key importance to the derivation of a polymerization distribution for a synthetic system that is continually producing new molecules with every generation. Previously, it has been assumed that for A. xylinum the number of polymers produced per cell is a constant, and that the first-order rate equation for the increase in the number of cells with time could be used in conjunction with the polymer-cell proportionality to predict the total number of polymeric molecules isolated at any time (5). This expression for $n_1(t)$, the total number of polymers isolated after time t , is written as:

$$n_1(t) = N_0 K e^{\alpha t}, \quad (5)$$

where N_0 is the original number of cells, K is equal to the number of polymers per cell, and α is the same first-order rate constant defined earlier. However, Eq. (5) only assumes that the number of polymers isolated is always proportional to the number of cells present. The definition of the total number of polymers present in any sample is the ratio of the mass to the number-average molecular weight (24). If \bar{M}_n is the number-average for the isolated bacterial cellulose then the number of polymers that have been isolated is

$$n_1(t) = w_0 k_i A e^{\alpha t} / \bar{M}_n, \quad (6)$$

where A is Avogadro's number. Because Eq. (5) and (6) are only equal when their coefficients ($N_0 K$) and ($w_0 k_i A / \bar{M}_n$) are also equal, the implication is that \bar{M}_n must be assumed constant with time. However, for a synthetic material that is increasing in average molecular weight this assumption cannot be made.

An alternative assumption is that \bar{M}_n varies with time, similar to Eq. (5), by the empirical function

$$\overline{M}_n(t) = K' e^{\alpha' t}, \quad (7)$$

where K' and α' are analogous, respectively, to K and α . Equation (6) may then be rewritten as

$$n_i(t) = n_0 e^{(\alpha - \alpha')t} = n_0 e^{\beta t}, \quad (8)$$

where $n_0 = w_0 k_i A / K'$, and the symbol $\beta = \alpha - \alpha'$ is the rate constant for the increase in the number of polymers with synthesis time, which - depending upon the value of α' - will not generally be equal to α . Therefore, no simplifying assumptions can be made about the rate constants for the increase in the number of polymers and the mass of cellulose with time. Both α and β will have to be measured experimentally.

To measure α , a series of cultures initiated at the same time but harvested at various intervals is required. From these samples either the number of cells, total dry mass, or yield of isolated cellulose has to be obtained. Plotting the logarithm of these parameters against the elapsed time between initiation and harvesting results in a straight line. For the yield of isolated cellulose, $w_{ic}(t)$, this line would be described by

$$\ln w_{ic}(t) = \ln (w_0 k_i) + \alpha t. \quad (9)$$

Thus, the slope of such a line is a measure of the rate constant.

To measure β , it is necessary to obtain both the yield and the number-average molecular weight \overline{M}_n . Thus, plotting the logarithm of the number of polymers against time will also result in a straight line with β as its slope if Eq. (8) is valid.

$$\ln n_i(t) = \ln (w_{ic}(t) / \overline{M}_n) = \ln n_0 + \beta t \quad (10)$$

In much the same manner as Eq. (4), a doubling or generation time for the number of polymers, τ_{poly} , can be defined:

$$\tau_{\text{poly}} = \ln 2 / \beta = \delta / \beta, \quad (11)$$

where $\delta = \ln 2$. The parameter τ_{poly} can be used to define a new dimensionless time parameter γ in units of generation or doubling time,

$$\gamma = t / \tau_{\text{poly}} = \beta t / \delta, \quad (12)$$

that permits a simplified notation for the kinetic expressions. Equation (8) can be restated simply as

$$n_i(t) = n_0 e^{\beta t} = n_0 e^{\delta \gamma} = n_0 2^\gamma. \quad (13)$$

The number of polymers that are introduced at any given time γ' between 0 and γ will be:

$$d(n_i(t)) / d\gamma' = n_0 \delta e^{\delta \gamma'}. \quad (14)$$

Those polymers introduced at γ' will polymerize for a duration of $\gamma - \gamma'$.

If polymerization proceeds through the sequential addition of cellulose precursors having a degree of polymerization equal to z , then the molecular weight of a polymer introduced as a precursor with k number of further additions is

$$M_k = x z (k + 1) \quad (15)$$

where $x = 162$, the molecular weight of anhydroglucose.

The percentage of polymers introduced at γ' with k number of polymerization events after $\gamma - \gamma'$, according to a Poisson probability function, is given as

$$p(k, \lambda, (\gamma - \gamma')) = e^{-\lambda(\gamma - \gamma')} (\lambda(\gamma - \gamma'))^k / k!, \quad (16)$$

where λ is the constant, average rate that at which event will occur per generation.

The combined products of Eq. (14) and Eq. (16) for every value of γ' yield the number distribution for a Poisson probability function and a first-order polymer introduction rate:

$$n(k, \lambda, \gamma) = n_0 p(k, \lambda, \gamma) + n_0 \delta \int_0^{\gamma} e^{\delta \gamma'} p(k, \lambda, (\gamma - \gamma')) d\gamma' \quad (17)$$

which, upon integration, yields:

$$n(k, \lambda, \gamma) = n_0 e^{-\lambda \gamma} \frac{(\lambda \gamma)^k}{k!} + \frac{n_0 \lambda^k \delta e^{\delta \gamma}}{(\delta + \lambda)^k + 1} \left[1 - e^{-(\delta + \lambda) \gamma} \sum_{i=0}^k \frac{((\delta + \lambda) \gamma)^i}{i!} \right]. \quad (18)$$

The number distribution is utilized to define the polymeric mass and the number-average and the weight-average degrees of polymerization, respectively:

$$w(\lambda, \gamma) = \sum_{k=0}^{\infty} M_k n(k, \lambda, \gamma) = x z u_1(\lambda, \gamma), \quad (19)$$

$$\overline{DP}_n(\lambda, \gamma) = (1/x) \sum_{k=0}^{\infty} M_k n(k, \lambda, \gamma) / \sum_{k=0}^{\infty} n(k, \lambda, \gamma) = z u_1(\lambda, \gamma) / u_0(\lambda, \gamma), \quad (20)$$

and

$$\overline{DP}_w(\lambda, \gamma) = (1/x) \sum_{k=0}^{\infty} M_k^2 n(k, \lambda, \gamma) / \sum_{k=0}^{\infty} M_k n(k, \lambda, \gamma) = z u_2(\lambda, \gamma) / u_1(\lambda, \gamma), \quad (21)$$

where the function, with $j = 0, 1, 2$,

$$u_j(\lambda, \gamma) = \sum_{k=0}^{\infty} (k + 1)^j n(k, \lambda, \gamma), \quad (22)$$

is referred to as the j 'th moment of the number distribution.

As can be seen, z has the role of a simple scaling factor and can be computed directly once the moments $u_0(\lambda, \gamma)$, $u_1(\lambda, \gamma)$, and $u_2(\lambda, \gamma)$ have been determined. However, in order to be able to calculate all three moments, it is necessary to first determine the average occurrence rate, λ . This is accomplished by solving

simultaneously the polydispersity relationship, which is independent of z , for several values of γ . Thus,

$$\overline{DP}_w(\lambda, \gamma) / \overline{DP}_n(\lambda, \gamma) = u_2(\lambda, \gamma) u_0(\lambda, \gamma) / u_1^2(\lambda, \gamma). \quad (23)$$

In summary, the experimental procedure suggested by the above theoretical derivation is:

- a) Measure yield and the molecular weight averages (w_1 , \overline{DP}_n , and \overline{DP}_w) as functions of time t .
- b) If \overline{DP}_n is constant, calculate α according to Eq. (9). If \overline{DP}_n is time-dependent, calculate β according to Eq. (10).
- c) Calculate τ_{poly} and convert all time values from t to γ .
- d) Determine the constant λ from a simultaneous solution of Eq. (23) for all values of γ .
- e) Finally, calculate z from Eq. (19)-(21).

EXPERIMENTAL

ACETOBACTER XYLINUM CULTURES

CULTURE CONDITIONS

Acetobacter xylinum, ATCC strain number 12733, was grown in a sterile liquid medium consisting of 20 g/L glucose, 5 g/L Bactopeptone, 5 g/L yeast extract, 2.7 g/L disodium phosphate, and 1.15 g/L citric acid at a pH of 6.0 throughout the duration of the experiments. Rapid growth rates were maintained by pipetting 2 mL of a 24-hr-old culture into a 250-mL Erlenmeyer flask containing 50 mL of medium at 30°C. All inoculation cultures were kept under a constant 125 cycle/min agitation to ensure sufficient homogeneity in the aliquots removed for inoculation.

GROWTH AND HARVEST OF BACTERIAL CELLULOSE

Six 4000-mL Erlenmeyer flasks were each filled with 450 mL of the liquid medium described above for a surface-to-volume ratio of 0.7 cm^{-1} . Every flask was simultaneously inoculated with 2 mL of inoculum from a 24-hr inoculation culture. Cultures were grown at 30°C with no agitation. Each flask was harvested after at least 72 hr of growth by draining the cellulose pellicles on a coarse, sintered-glass filter, storing overnight in a deep-freeze, and then freeze-drying.

PURIFICATION OF BACTERIAL CELLULOSE

The freeze-dried pellicle was added to 100 mL of 2% nitrogen-saturated NaOH and autoclaved for 60 min at 121°C. The alkali containing the cellular debris was drained and replaced with 1% acetic acid, and then placed on a shaker for 4 hr. After this period, fresh acid was added and the shaking continued overnight. The acid was finally replaced with distilled water which was also replaced every 24 hr for three days. Finally, after freeze-drying, the purified cellulose was measured on an analytical balance for its yield.

DETERMINATION OF THE MOLECULAR WEIGHT AVERAGES

PREPARATION OF CELLULOSE TRICARBANILATES

Due to the difficulty involved in directly carbanilating dried bacterial cellulose, the methylol derivative was first prepared in DMSO (25). An oil bath, maintained at 120°C by a combination hot plate-stirrer, was used to prepare the methylol cellulose derivatives. The oil bath consisted of a pyrex, 120 mm x 60 mm, crystallizing dish filled with silicone fluid. A 2-inch Teflon-covered magnetic stirring bar was utilized to minimize the formation of temperature gradients, and the temperature was controlled to within $\pm 0.5^\circ\text{C}$ with the aid of an immersion probe connected to the hot plate.

The cellulose/DMSO solutions were prepared by initially mixing the bacterial cellulose with 40 mL of DMSO in 100-mL beakers which were suspended in the silicone bath directly above the stirring bar. The size of these beakers was selected to allow the level of the DMSO to be sufficiently below the level of the silicone fluid in order to prevent the occurrence of polyoxymethylene films that would form if the surface of the solutions were allowed to cool excessively. A 1-inch Teflon covered stirring bar was added to the mixture and operated by induction from the larger stirring bar in the silicone fluid. The temperature of the mixture was monitored by a suspended thermometer. Once the mixture had attained the same temperature as the bath, the thermometer was removed and 2.4 g of paraformaldehyde (Tridom Chemical Co.) was added directly to the mixture. This addition produced the immediate result of formaldehyde gas evolution accompanied by the dissolution of the cellulose to produce a clear solution. After complete dissolution, the methylol cellulose solution was heated until no further gas evolution could be observed. The solution was then transferred to a dropping funnel.

The bacterial cellulose was regenerated by slowly introducing the methylol cellulose solution from the dropping funnel into 200 mL of a 5% sodium sulfite solution (10 g Na_2SO_3 /200 mL H_2O). The aqueous regeneration solution was kept at 80°C and constantly stirred to facilitate rapid formation of the soluble formaldehyde-sulfite addition complex (26). After 20 min the cellulose mixture was centrifuged, decanted, and washed with five 100-mL aliquots of distilled H_2O . Freeze-drying subsequently produced a highly divided foamlike material. A cotton-linter cellulose (Hercules' type N-30, $\overline{\text{DP}}_n = 816$) that could be derivatized without this procedure demonstrated a $\overline{\text{DP}}_n = 797$ after treatment, indicating very little degradation.

After freeze-drying, 4.6 mg to 62.4 mg of bacterial cellulose was placed into a 100-mL narrow-necked, screw-top derivatization bottle and vacuum desiccated overnight. Into this bottle, 100 mL of pyridine and 7.2 mL of phenylisocyanate were then pipetted, after which the bottle was sealed with a Teflon-lined cap. The bottles were then placed in a reactor vessel that was kept slowly revolving at 80°C for 48 hr in an oil bath (46). After completion of the reaction, 3.3 mL of methanol was added to each bottle to destroy any excess phenylisocyanate.

The derivative was precipitated by mixing the pyridine solution with 130 mL of dioxane and slowly dripping this new mixture into a rapidly stirring solution of 800 mL methanol and 5 mL glacial acetic acid in a 2000-mL beaker. After all the pyridine-dioxane solution had been added, the tricarbanilate was allowed to settle overnight. The methanol solution was then siphoned off, and the precipitate was collected in a centrifuge bottle. The precipitate was then washed in succession with mixtures of 200 mL of methanol and 1.25 mL glacial acetic acid, 200 mL of distilled H_2O and 1.25 mL glacial acetic acid, and finally washed with 200 mL of distilled H_2O . The precipitate was then freeze-dried.

GPC ANALYSIS

To prepare the samples for gel permeation chromatography, approximately 9 mg of bacterial cellulose tricarbanilate was dissolved in 5 mL of stabilized tetrahydrofuran (THF). To this solution, 0.03 mL of a 2.5% THF solution of methyl-N-phenylcarbamate was added as a low molecular weight, total-permeation internal standard. Styragel columns (Waters Associates) having permeability ranges of 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 nm were used to obtain the chromatograms for the bacterial cellulose tricarbanilates. The elution solvent was purified THF (51), the elution rate was 2 mL/min, and the spectrophotometric detector was operated at 235 nm. A dispersion-compensated universal calibration, utilizing a linear polystyrene calibration curve and cellulose tricarbanilate standards, was used to calculate the number-average and weight-average degrees of polymerization. This calibration procedure is described in detail in Appendix II.

The chromatograms were digitalized at 2-mL intervals and analyzed by numerical integration techniques for the molecular weight averages. McCrackin's computer program GPC was utilized for this analysis (27). Base lines were justifiably assumed to be linear across the first and last points entered in the program and were automatically subtracted from the data set.

RESULTS AND DISCUSSION

The moments of the number distribution for a Poisson polymerization function, as defined by Eq. (21), have definite physical interpretations. The first moment, $u_1(\lambda, \gamma)$, is proportional to the predicted yield $w(\lambda, \gamma)$ as demonstrated by Eq. (18), and the zeroth moment $u_0(\lambda, \gamma)$ is proportional to the predicted number of polymers $w(\lambda, \gamma)/\overline{DP}_n(\lambda, \gamma)$, from Eq. (19) and Eq. (20). The second moment $u_2(\lambda, \gamma)$, which is equal to the product of $w(\lambda, \gamma)$ and $\overline{DP}_w(\lambda, \gamma)$ from Eq. (19) and (21), corresponds to the manner in which the polymers are spread about the average value and can thus be regarded as a measure of the width of the distribution.

The actual experimental values of $w_{ic}(\gamma)$, $\overline{DP}_n(\gamma)$, and $\overline{DP}_w(\gamma)$, measured at different synthesis times γ , can also be used to define experimental moments $v_j(\gamma)$ analogous to the Poisson distribution moments $u_j(\lambda, \gamma)$, where

$$v_0(\gamma) = w_{ic}(\gamma)/\overline{DP}_n(\gamma), \quad (24)$$

$$v_1(\gamma) = w_{ic}(\gamma), \quad (25)$$

and

$$v_2(\gamma) = w_{ic}(\gamma) \overline{DP}_w(\gamma). \quad (26)$$

These experimental moments would then be proportional to $u_j(\lambda, \gamma)$ if the Poisson-predicted values of $w(\lambda, \gamma)$, $\overline{DP}_n(\lambda, \gamma)$, and $\overline{DP}_w(\lambda, \gamma)$ were equal to the experimental values of $w_{ic}(\gamma)$, $\overline{DP}_n(\gamma)$, and $\overline{DP}_w(\gamma)$. Thus, by substituting Eq. (24)-(26) into Eq. (19)-(21):

$$v_0(\gamma) = x u_0(\lambda, \gamma), \quad (27)$$

$$v_1(\gamma) = x z u_1(\lambda, \gamma), \quad (28)$$

and

$$v_2(\gamma) = x z^2 u_2(\lambda, \gamma). \quad (29)$$

What must now be demonstrated is whether or not unique values of λ and z can be obtained to satisfy Eq. (27)-(29).

The results of four experiments determining the yield, $w_{ic}(t)$, and the degrees of polymerization, $[\overline{DP}_n(t) \text{ and } \overline{DP}_w(t)]$, from six, simultaneously inoculated cultures are presented in Table I. In agreement with previous results (4,5), the yields for experiments No. 7, 9, and 10 demonstrated first-order kinetics. By utilizing Eq. (9) as a linear regression model, the first-order rate constant α can be calculated (Table II) for each experiment, and the three sets of data can be plotted colinearly as functions of $\ln(w_{ic}/w_0)$ vs. t/τ_{cell} as in Fig. 1. Experiment No. 8 did not demonstrate linearity because it became contaminated with a competing organism during inoculation and apparently entered its death phase prematurely. The first six experiments were preliminary in nature and were utilized to establish inoculation and cellulose derivatization techniques.

The weight-average DPs increased with time, as had been previously reported, until about 95 hr, where a decrease in these values occurred. However, such decreases are not attributed to degradation, since the continued introduction of newer, low-molecular weight material will gradually offset the effects of the higher molecular weight material and reduce the weight-average DP (4).

For experiments No. 7, 9, and 10 the number of polymers $[n_i(t) = w_{ic}(t)/\overline{DP}_n(t)]$ also demonstrated first-order kinetics. The first-order rate constants, β , for these experiments were calculated using Eq. (10) and are represented together with the respective rate constants, α , in Table II. What is notable is that the values of α for each experiment are greater than the values for β , justifying the precautions taken in Eq. (8). The significance of α being greater than β is that the effective number of polymers per cell is not constant, but rather, decreases with time as can be seen by

$$n/N = (n_0/N_0) e^{(\beta-\alpha)t}, \quad (30)$$

where n is the number of polymers, and N , the number of cells.

TABLE I
EXPERIMENTAL DATA

Expt.-Samp.	t(hr)	w _i (mg)	\overline{DP}_n	\overline{DP}_w	$\overline{DP}_w/\overline{DP}_n$
7-1	72.57	6.1	1310	2510	1.92
7-2	78.35	7.7	1270	2730	2.14
7-3	84.55	9.4	1190	3040	2.56
7-4	90.82	14.5	1690	5130	3.04
7-5	96.60	19.0	1470	4490	3.05
7-6	102.55	22.3	1290	3320	2.57
8-1	72.07	4.6	905	2240	2.48
8-2	77.73	8.5	976	2770	2.84
8-3	85.08	10.6	1170	3850	2.44
8-4	90.37	11.3	1250	3740	3.01
8-5	95.37	12.4	1200	3690	3.07
8-6	101.65	12.5	1080	2480	2.30
9-1	71.20	8.6	1360	3850	2.84
9-2	74.03	10.9	1490	4410	2.95
9-3	75.92	13.1	1650	5510	3.34
9-4	77.95	12.8	1650	5647	3.42
9-5	80.73	19.8	1720	5770	3.36
9-6	82.63	34.1	2210	7290	3.30
10-1	73.35	8.4	1100	3440	3.13
10-2	77.73	15.5	1670	5360	3.21
10-3	93.03	36.4	1210	4520	3.75
10-4	95.10	30.7	1670	6000	3.60
10-5	97.23	45.1	1700	6010	3.53
10-6	101.13	62.4	1350	5470	4.04

TABLE II

DETERMINATION OF FIRST-ORDER RATE CONSTANTS α AND β

Expt.	Slope	Intercept	Corr. Coef.
7	$\alpha = 0.046$	$w_0 = 217.0$	$r = 0.993$
	$\beta = 0.042$	$n_0 = 0.215$	$r = 0.988$
9	$\alpha = 0.110$	$w_0 = 2.77$	$r = 0.974$
	$\beta = 0.076$	$n_0 = 0.0271$	$r = 0.981$
10	$\alpha = 0.067$	$w_0 = 73.3$	$r = 0.990$
	$\beta = 0.063$	$n_0 = 0.0756$	$r = 0.981$

In Table III, the empirical moments $v_j(\gamma)$ calculated from the experimental data are presented for experiments No. 7, 9, and 10 along with the values of γ calculated by multiplying t with $\beta/\ln 2$. Table IV documents that for each culture, all three moments demonstrate at least approximate first-order kinetics. This was expected for $v_0(\gamma)$ and $v_1(\gamma)$, but the first-order behavior for $v_2(\gamma)$ further emphasizes the inherent appropriateness of monitoring the extent of polymerization through the second empirical moment rather than \overline{DP}_w , since the conflicting effects of increasing polymer mass are avoided. Expressed in time units of γ , the slopes for $v_0(\gamma)$ vs. γ are approximately equal to the constant $\delta = \ln 2$. The slopes for the $v_1(\gamma)$ and $v_2(\gamma)$ relationships, however, are not equal from one experiment to the next, but for each experiment, the magnitude of the slopes for $v_0(\gamma)$, $v_1(\gamma)$, and $v_2(\gamma)$ increases in that respective order. These results indicate that, based on the rate of polymer introduction [$v_0(\gamma)$ vs. γ], the rate of mass accumulation [$v_1(\gamma)$ vs. γ] and the relative polymer elongation rate [$v_2(\gamma)$ vs. γ] can vary from condition to condition and are apparently not identically synchronized with the cellulose replication rate. Figure 2 depicts the moments $v_1(\gamma)$ as a function of γ , and Fig. 3 depicts the $v_2(\gamma)$ -vs.- γ relationship for experiments No. 7, 9, and 10.

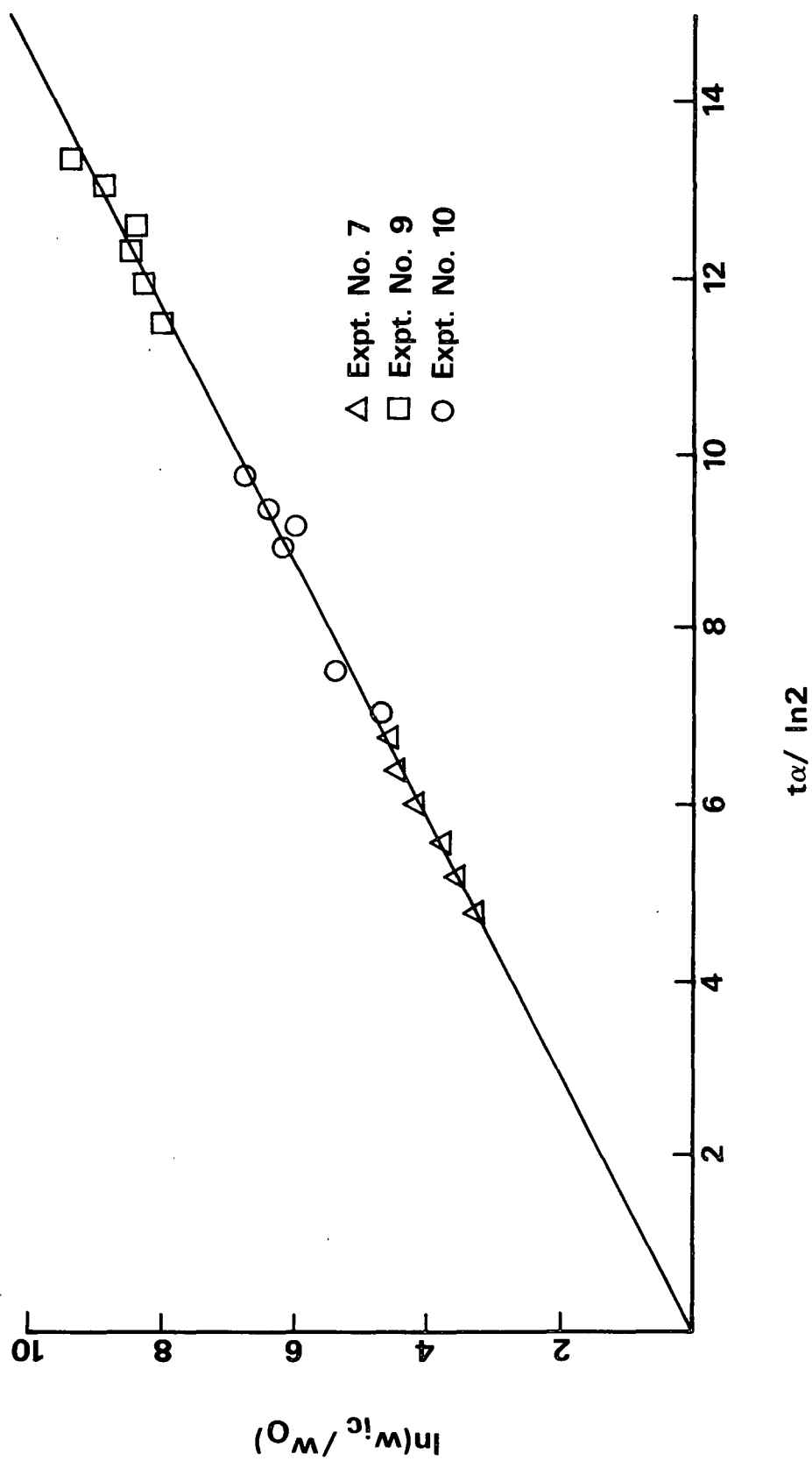


Figure 1. Yields of isolated bacterial cellulose from cultures harvested after 72 hours of synthesis.

TABLE III
EMPIRICAL MOMENTS FROM EXPERIMENTAL DATA

Expt.-Samp.	γ	$v_0(\gamma)$	$v_1(\gamma) \times 10^{-3}$	$v_2(\gamma) \times 10^{-7}$
7-1	4.42	4.66	6.1	1.35
7-2	4.77	6.04	7.7	2.10
7-3	5.15	7.91	9.4	2.86
7-4	5.53	8.59	14.5	7.44
7-5	5.88	12.9	19.0	8.53
7-6	6.24	17.3	22.3	7.40
9-1	7.78	6.34	8.6	3.31
9-2	8.09	7.31	10.9	4.80
9-3	8.30	7.94	13.1	7.22
9-4	8.52	7.76	12.8	7.23
9-5	8.83	11.5	19.8	11.43
9-6	9.03	15.4	34.1	24.85
10-1	6.62	7.66	8.4	2.89
10-2	7.02	9.29	15.5	8.31
10-3	8.40	30.2	36.4	16.44
10-4	8.59	18.4	30.7	18.42
10-5	8.78	26.5	45.1	27.12
10-6	9.13	46.1	62.4	34.12

It is now obvious from the semilogarithmic forms of Eq. (27)-(29) that the slopes for $u_j(\lambda, \gamma)$ would be equal to the slopes for $v_j(\gamma)$ if the experimental results were, in fact, a consequence of a random Poisson polymerization process. Furthermore, the intercepts for $u_1(\lambda, \gamma)$ and $u_2(\lambda, \gamma)$ would differ from the intercepts of $v_1(\gamma)$ and $v_2(\gamma)$ by the values of $\ln z$ and $\ln z^2$, respectively. Thus, the desired value for λ

has to satisfy a severe slope and intercept criterion. Crucial to this whole question is whether or not the functions of $u_j(\lambda, \gamma)$ are linear on a semilogarithmic basis. Figure 4 demonstrates for $\lambda = 1$, that $u_0(\lambda, \gamma)$ has a constant slope equal to $\delta = \ln 2$, as expected, but that $u_1(\lambda, \gamma)$ and $u_2(\lambda, \gamma)$ only approximate linearity at values of γ greater than 3 generations. In this linear range, however, the slopes of the functions $u_1(\lambda, \gamma)$ and $u_2(\lambda, \gamma)$ are practically equal to the slope for $u_0(\lambda, \gamma)$. But, because the experimental slopes for $v_j(\gamma)$ had significantly different values, a value of λ equal to unity will not be applicable to these values. Therefore, the possibility of whether or not a different value for λ could produce different values for the slopes of $u_1(\lambda, \gamma)$ and $u_2(\lambda, \gamma)$ has to be investigated.

TABLE IV

FIRST-ORDER KINETIC RELATIONSHIPS FOR $v_0(\gamma)$, $v_1(\gamma)$, AND $v_2(\gamma)$

Expt.	Moment	Slope	Intercept	Corr. Coef.
7	$v_0(\gamma)$	0.695	-1.54	0.988
	$v_1(\gamma)$	0.752	5.37	0.993
	$v_2(\gamma)$	1.280	10.80	0.973
9	$v_0(\gamma)$	0.690	-3.59	0.980
	$v_1(\gamma)$	1.020	1.06	0.972
	$v_2(\gamma)$	1.470	5.79	0.974
10	$v_0(\gamma)$	0.693	-2.58	0.981
	$v_1(\gamma)$	0.737	4.30	0.990
	$v_2(\gamma)$	0.870	11.70	0.965

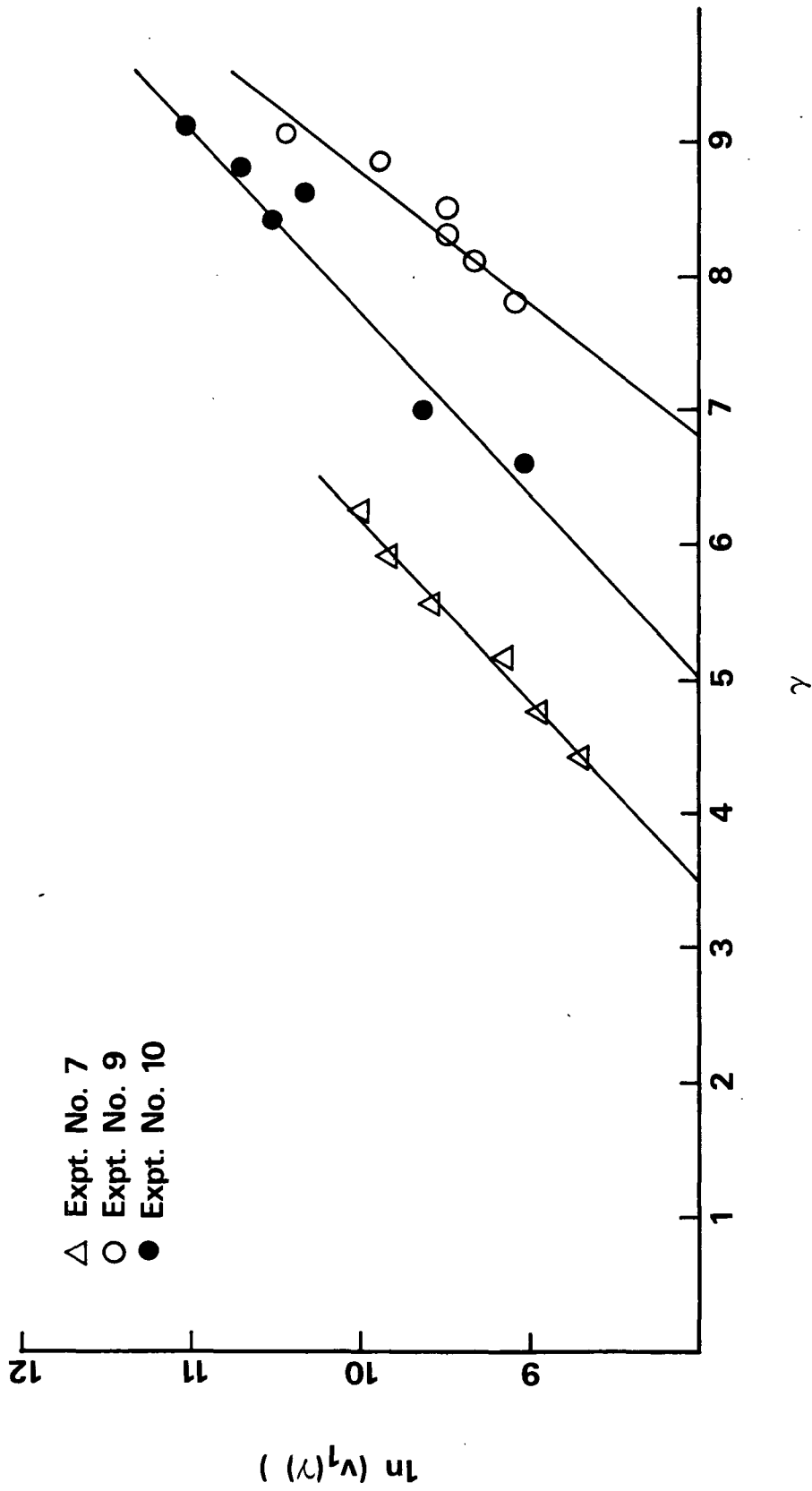


Figure 2. Empirical first-moment as a function of polymer-number generation time.

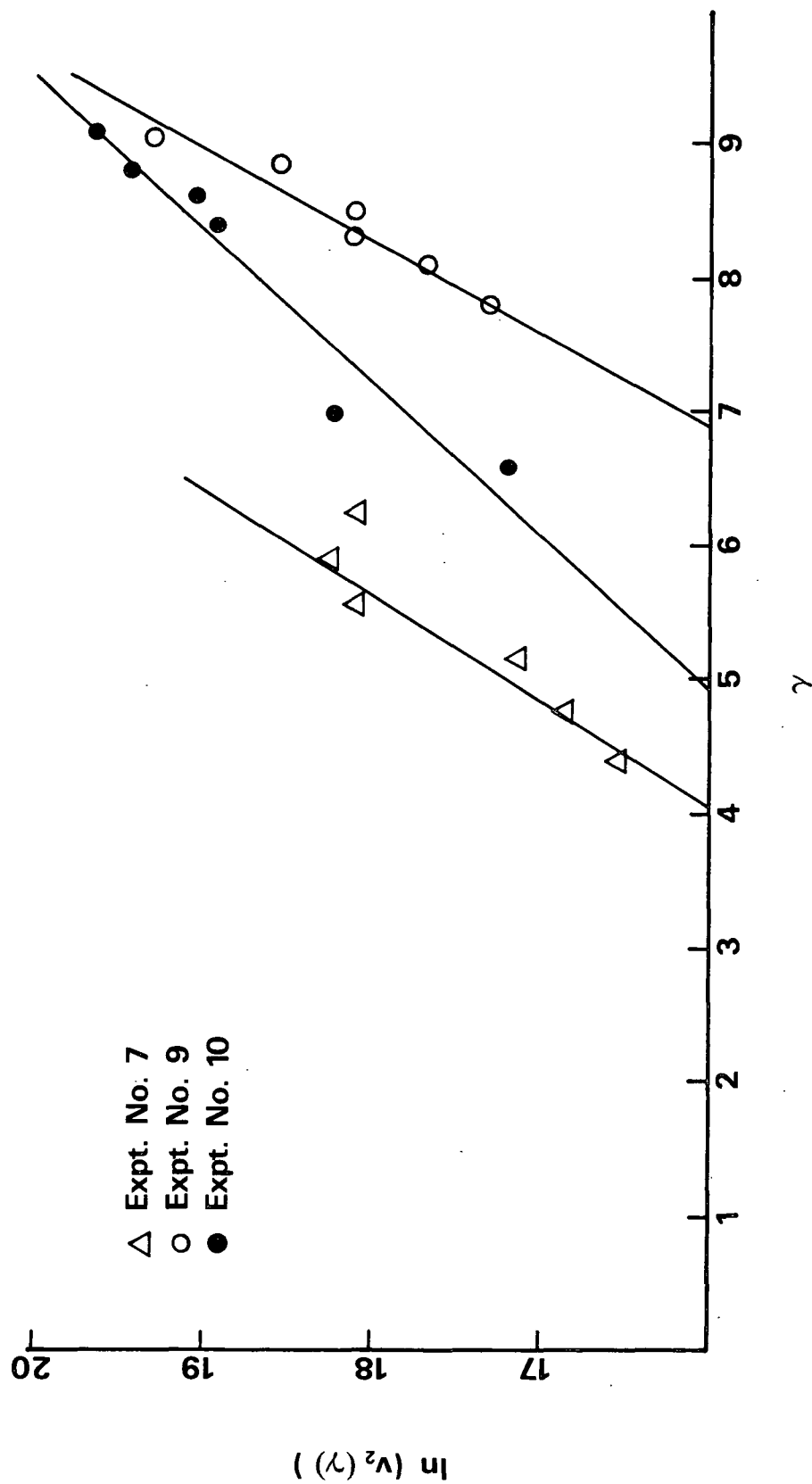


Figure 3. Empirical second-moment as a function of polymer-number generation time.

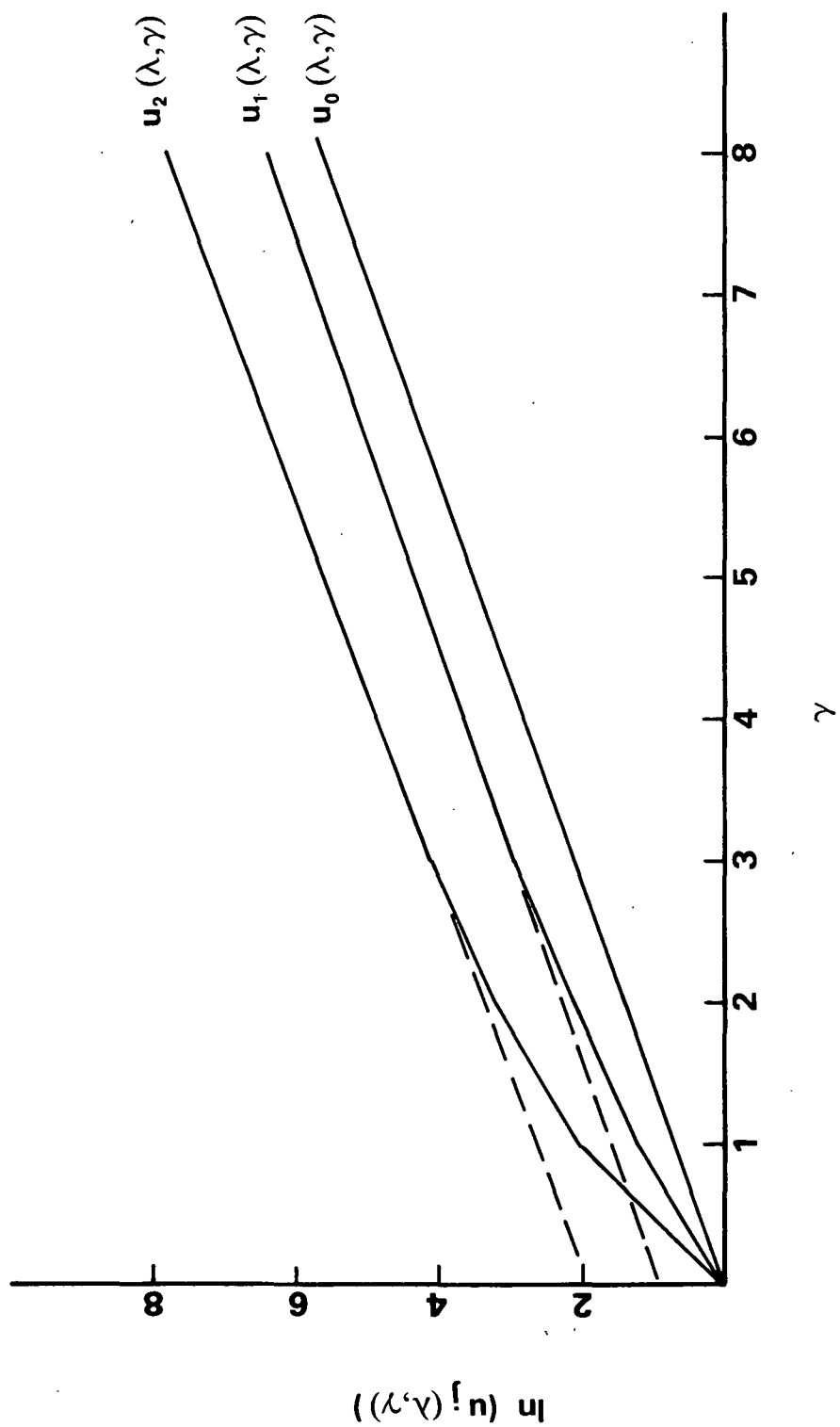


Figure 4. Theoretical Poisson-moments: $u_0(\lambda, \gamma)$, $u_1(\lambda, \gamma)$, and $u_2(\lambda, \gamma)$, where $\lambda = 1$.

The functions $u_1(\lambda, \gamma)$ and $u_2(\lambda, \gamma)$ vary directly in value with λ . Thus, the value of z will vary inversely with λ , since larger $u_1(\lambda, \gamma)$ and $u_2(\lambda, \gamma)$ values require a smaller correction to equal $v_1(\gamma)$ and $v_2(\gamma)$ through Eq. (28) and Eq. (29), respectively. Choosing values for λ less than unity will not significantly change the slope for either $u_1(\lambda, \gamma)$ or $u_2(\lambda, \gamma)$, but rather bring them closer to the limiting situation of superimposition with $u_0(\lambda, \gamma)$. But, increasing the value of λ does not, unfortunately, improve this situation. For $u_1(\lambda, \gamma)$, this phenomenon is depicted in Fig. 5. Apparently, the same limiting slope is attained for all values of λ . The fact that this is also the situation for $u_2(\lambda, \gamma)$ is demonstrated by examining the polydispersity relationship as a function of γ .

Figure 6 illustrates a family of curves for the theoretical polydispersity-vs.- γ relationship of the Poisson polymerization process, described by Eq. (23). Each curve is defined by a different value of λ . As the generation time γ increases, each curve increases in value until it reaches a constant value. As λ increases, the polydispersities at each value of γ assume greater values. However, as λ also increases, the polydispersities reach a constant value at earlier times. Since the rate of polymer introduction is identical for all these curves, a constant polydispersity can be regarded as a distribution equilibrium-state where the effects of both polymerization and polymer introduction counterbalance each other such that the relative shape of the distribution no longer changes. The empirical relationship between the equilibrium polydispersity π_e and its respective value of λ was found to be a hyperbola

$$(\pi_e - 2.000) (\lambda + \ln 1.994) = -\ln 1.999 \approx -\delta, \quad (31)$$

as demonstrated by Fig. 7. The maximum value of π_e , which corresponds to an infinitely large value for λ and an infinitely small value for the event-size z , is 2. Therefore, monomeric stochastic polymerization of isolatable cellulose cannot exceed

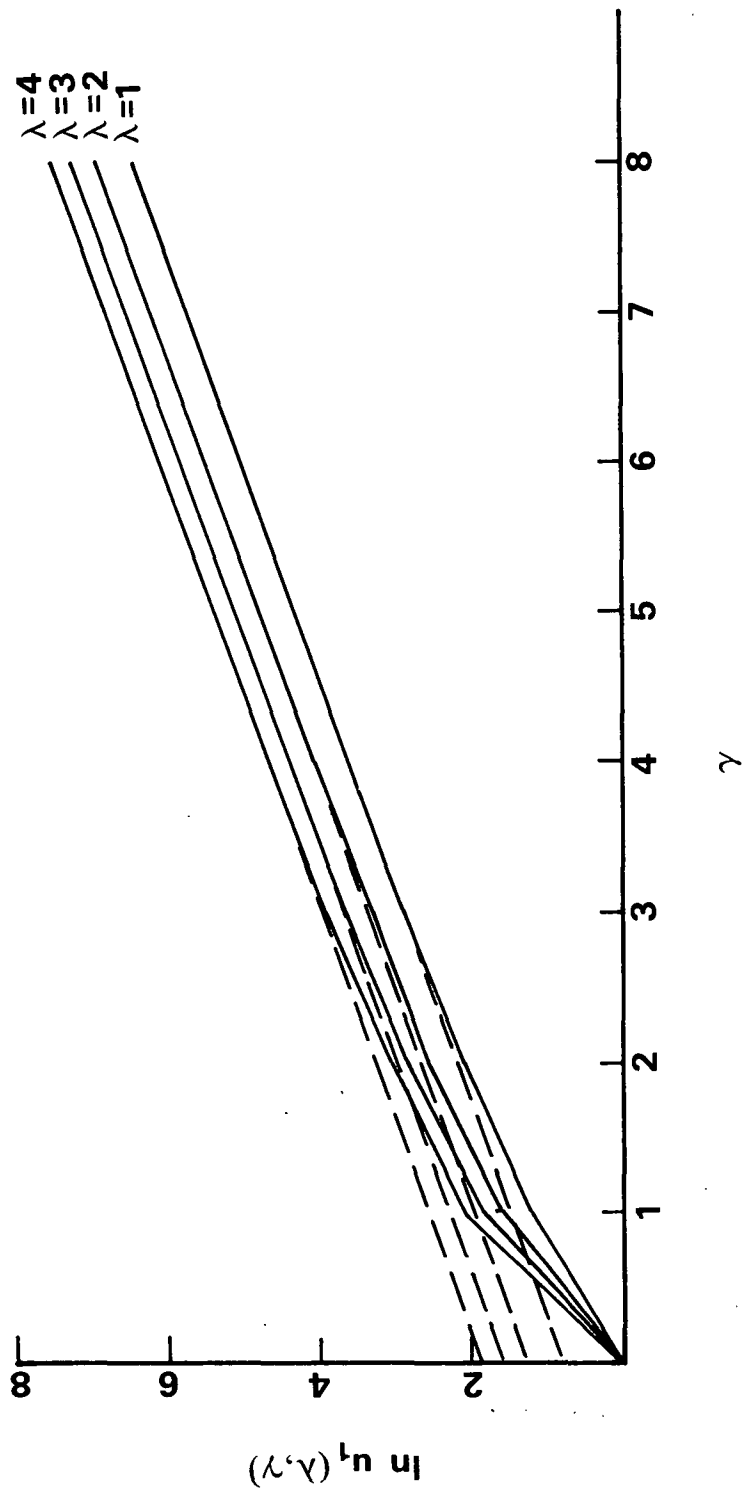


Figure 5. The theoretical Poisson-moment $u_1(\lambda, \gamma)$ as a function of generation time and λ .

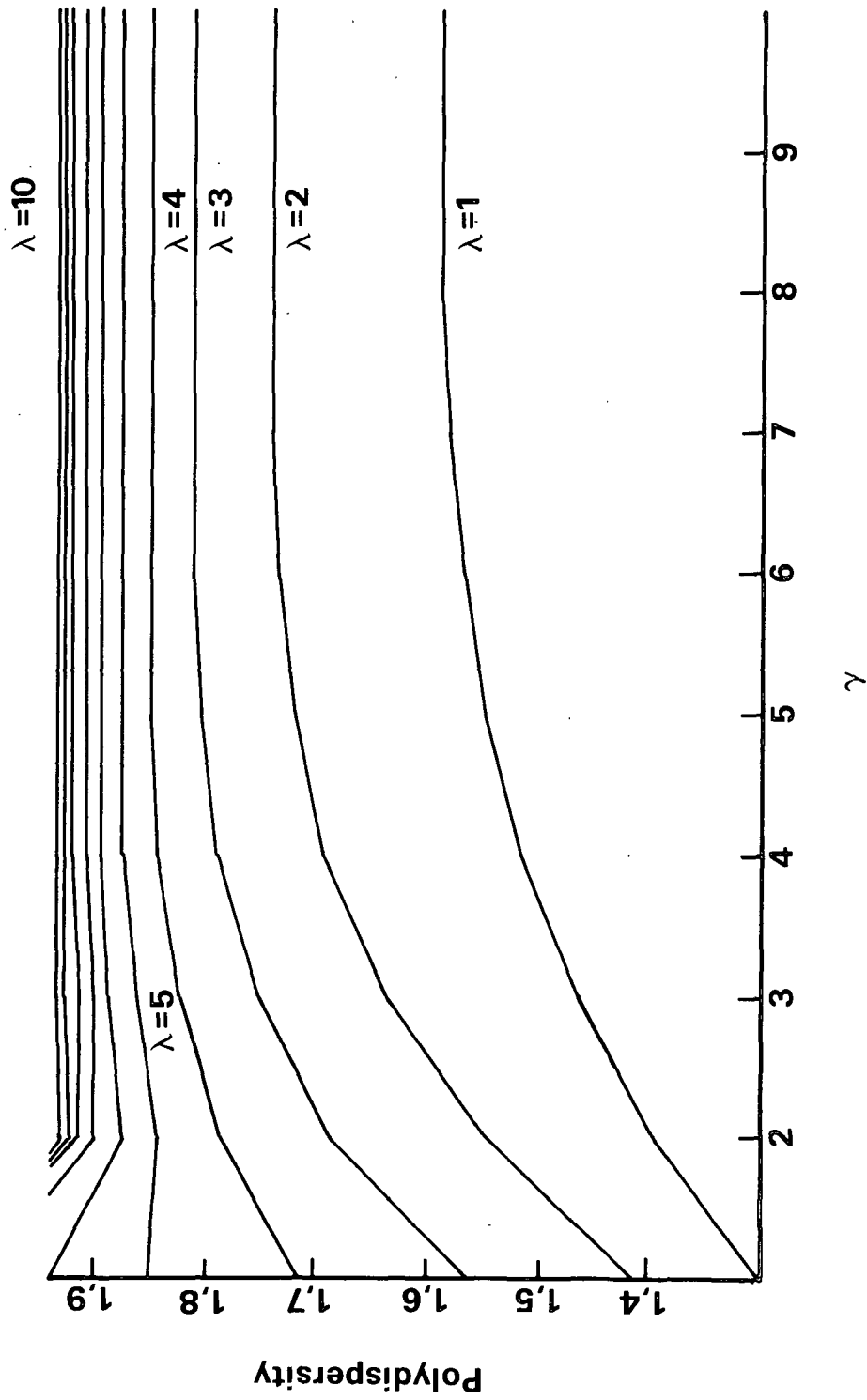


Figure 6. Theoretical Poisson polydispersity as a function of generation time γ , and event rate λ .

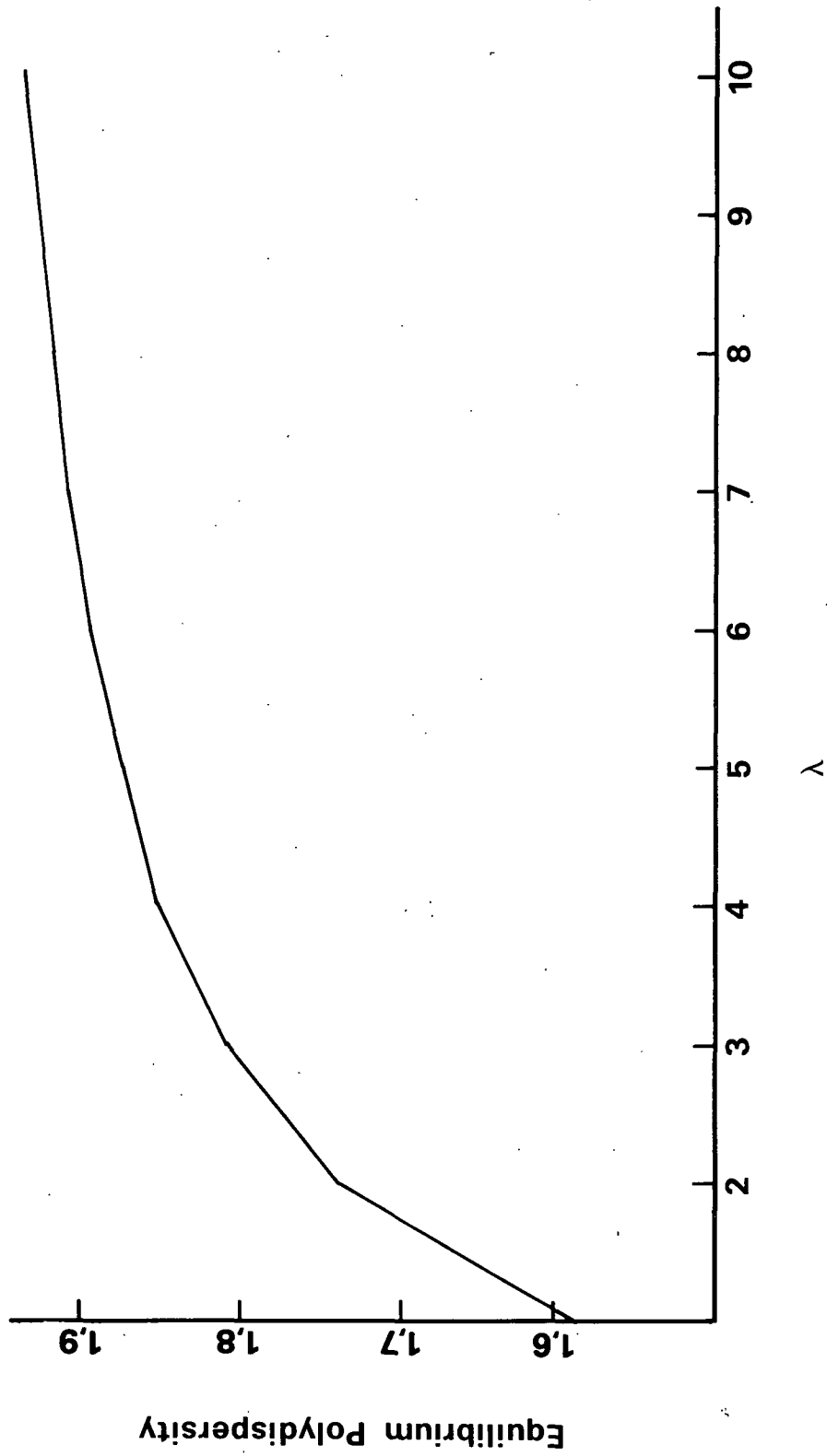


Figure 7. Theoretical Poisson equilibrium polydispersity vs. the event rate λ .

this polydispersity, while oligomeric polymerizations would have reduced values for π_e , dependent upon their respectively greater values of z and lesser values for λ . A value of $\lambda = 0$ would be indicative of no polymerization, only polymer introduction, and results in $\pi_e \approx 1$.

As is evidenced in Table I, the polydispersities for experiments No. 7, 9, and 10 are, with the exception of only one value, greater than 2. Because no value of λ exists that will produce this result for a Poisson polymerization mechanism, it must be assumed that an entirely different mechanism is operating for the production of isolatable bacterial cellulose. The rate of polymer introduction, defined by $u_0(\lambda, \gamma)$ -vs.- γ , is constant regardless of the value of λ since polymerization is an independent phenomenon. Hence, the polymerization-sensitive moments, $u_1(\lambda, \gamma)$ and $u_2(\lambda, \gamma)$, do not properly predict the observed polymerization distributions. The actual empirical moment for $v_2(\gamma)$ appears to have a value exceeding what is predicted by $u_2(\lambda, \gamma)$, thus accounting for the larger polydispersities. Therefore, it is questionable that isolated cellulose is the result of a polymerization process whereby each cellulose molecule experiences a random event that will increase its degree of polymerization. But, in order to prove that the Poisson function is not applicable to the growth process of isolated bacterial cellulose, it must first be shown that the GPC-derived polydispersities, after correction for peak dispersion effects, are, in fact, greater than 2 and not just values that have only been partially corrected.

For a linear calibration equation, the band spreading correction factor is a constant, symbolized as P (28). The polydispersity π of any chromatogram is determined approximately from its statistical width σ_v (the standard deviation with regard to elution volume) via the equation

$$\pi = P^2 \exp(D_2^2 \sigma_v^2), \quad (32)$$

where D_2 is the absolute value of the calibration equation's slope (see Appendix II). If the value of P is measured erroneously larger than its true value, P_t , then the resulting polydispersities, π , will be too large. Thus, to test the hypothesis that the corrected polydispersities are only partially corrected, the assumption is made that the lowest polydispersity, $\pi = 1.15$, which was obtained from the narrowest cellulose calibration standard utilized, was actually unity or perfectly monodisperse. The correction factor that was necessary to produce this result was

$$P_t^2 = 0.87 P^2. \quad (33)$$

However, the highest polydispersity that will be reduced by this factor to $\pi = 2$ or below is 2.3. Since the majority of polydispersities for the bacterial celluloses listed in Table I are above this value, it must be concluded that true polydispersities above 2 actually exist for the experimental samples. Therefore, a Poisson polymerization mechanism is not applicable for isolatable bacterial cellulose.

The significance of specifying isolated bacterial cellulose is that only experimentally isolated material can be analyzed for its molecular weight distribution. However, it may be hypothesized that although isolated cellulose does not demonstrate Poisson polymerization kinetics, the unaffected, total product may, because the isolation technique utilized may expose the total polymerization product to several possible degradation reactions that are functions of alkali (NaOH) concentration, oxygen concentration, temperature, and time. In alkali, cellulose will swell and become uniformly more accessible to degradation that is a direct function of all four of the above-mentioned variables. Thus, in order to minimize severe degradation, it is necessary to avoid oxygen and excessive temperatures while limiting the exposure of cellulose to the lowest practical concentration of alkali for the shortest period. In the presence of oxygen, chain cleavage will increase the total number of molecules while reducing their average length. As a result, the polydispersity

of cellulose undergoing oxidation rapidly decreases (29). In the absence of oxygen, rapid degradation will occur at temperatures above 140°C (30,31), but between this high temperature and 50°C, degradation is limited to the peeling reaction (32). Since peeling is expected to remove only as much as 68 glucose units per chain before the reaction is stopped (33), the polydispersity will be reduced only at a rate equal to the approximate decrease in \overline{DP}_w , as \overline{DP}_n tends to remain constant due to the very small change in the number of molecules (29). Finally, the removal of low molecular weight material through the various sample-handling procedures results in an increased \overline{DP}_n , which also results in a decrease of the polydispersity. Therefore, the only expected outcome of an alkaline isolation procedure is a reduction in the polydispersity, making it very unlikely that the relatively large polydispersities observed were actually increased from lower values due to the sample preparation procedures. Thus, it may be concluded that, for both the total polymerization product and the isolatable cellulose, a Poisson polymerization function does not apply.

Further evidence for this conclusion can be obtained from the examination of the GPC chromatograms of the bacterial cellulose cultures. Figure 8 depicts the six consecutive chromatograms for experiment No. 7. The observable growth pattern is that the greatest accumulation of mass occurs predominantly at the lower elution-volume values. Because elution volume in GPC is inversely related to the molecular weight, this noticeable mass accumulation corresponds to molecules with high molecular weights. When these same chromatograms are converted from a mass basis for their ordinate to a molar basis, as in Fig. 9, it becomes apparent that the greatest accumulation is due to relatively few, high molecular weight molecules. Therefore, the basic assumption for a Poisson mechanism - that all polymers have equal probabilities for further polymerization - was clearly demonstrated by both Fig. 8 and 9 not to be valid for the actual production of isolatable bacterial cellulose.

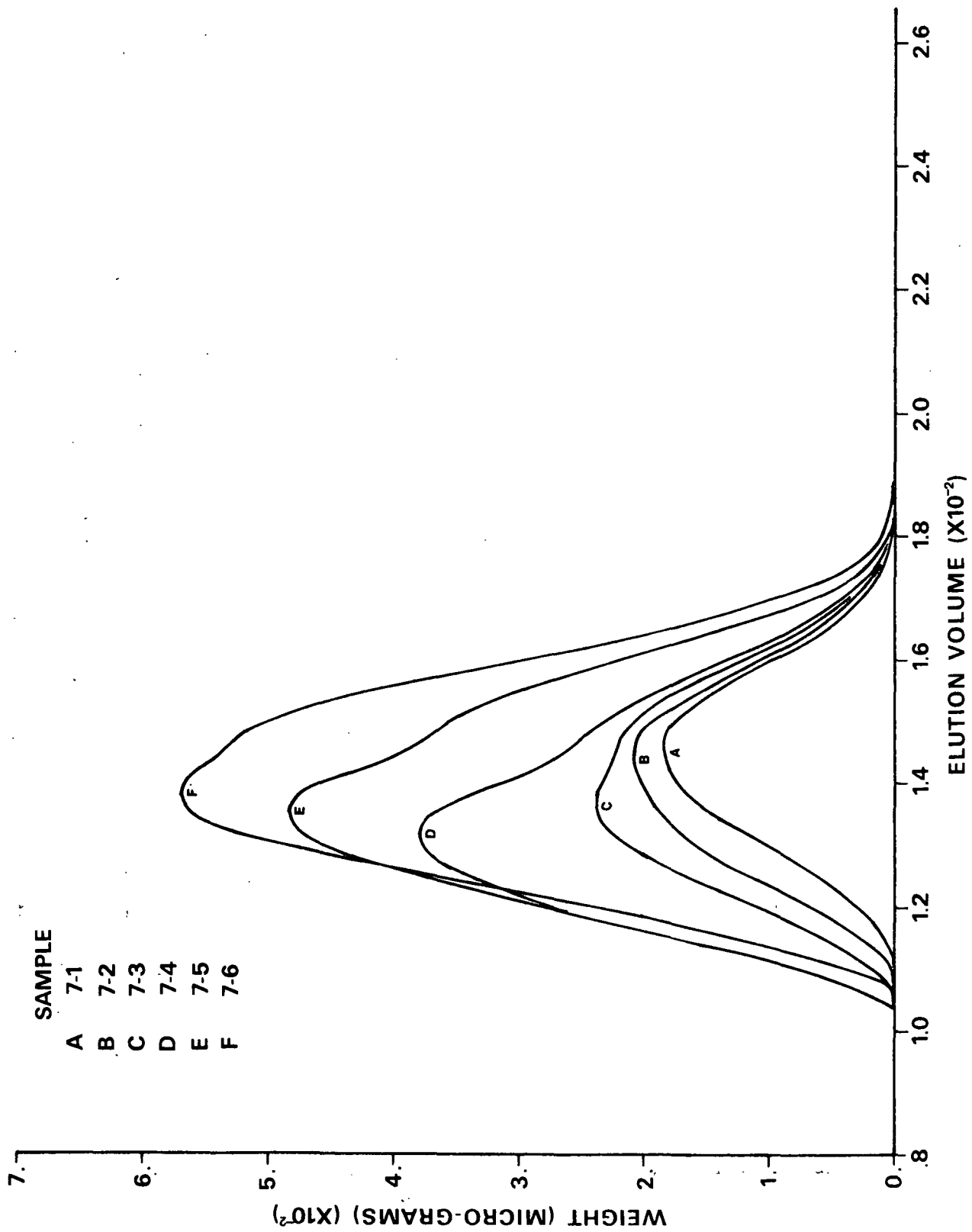


Figure 8. The yield normalized GPC chromatograms for Experiment Number 7 (weight-biased).

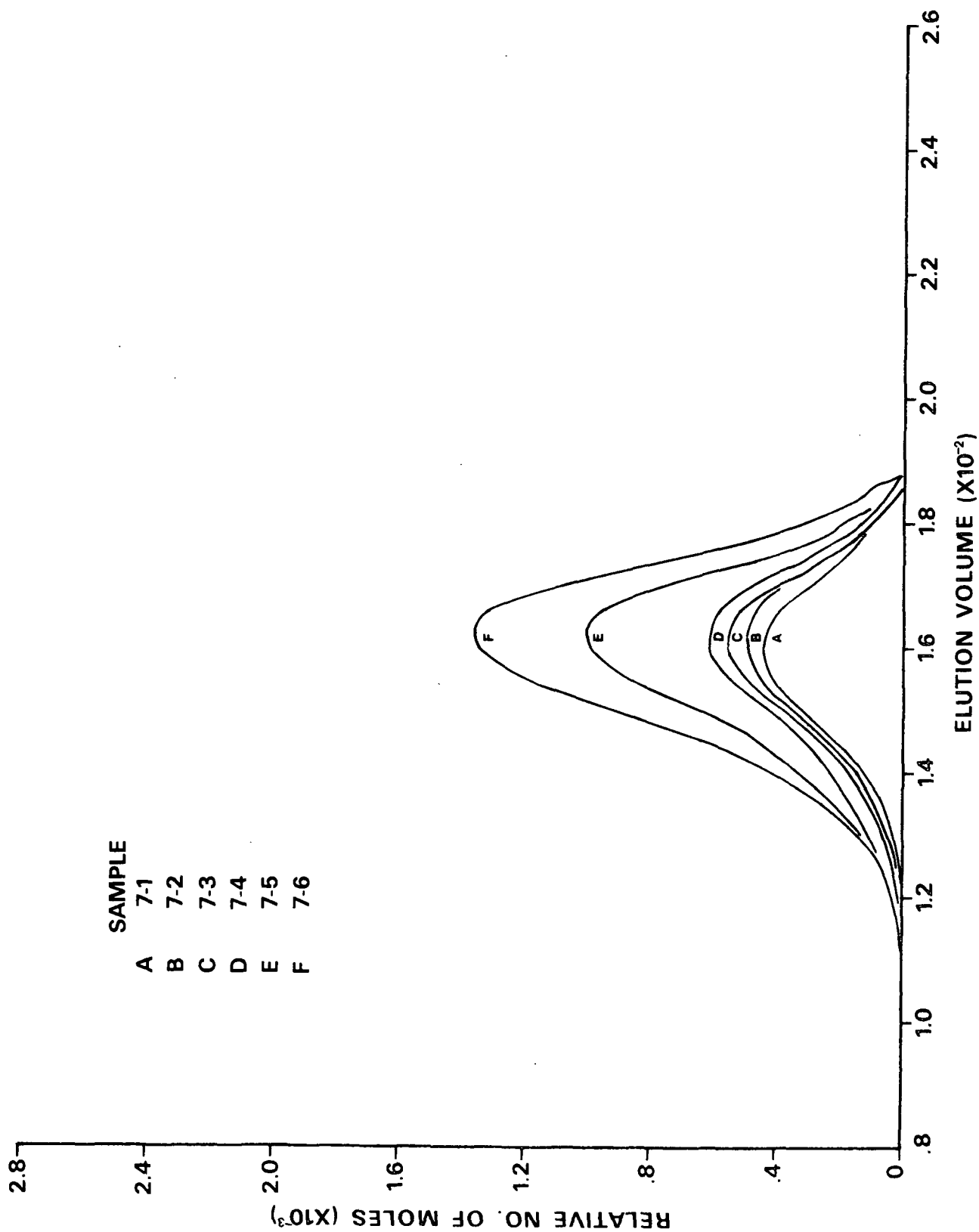


Figure 9. The GPC chromatograms for Experiment Number 7 with ordinate converted to mole-number.

In regard to the soluble, polymeric-precursor model proposed by Colvin and Leppard (7), the experimental conclusions discussed above are not sufficient to disprove the existence of polymeric precursors. It only suggests that they will bond preferentially to established chains in a fashion not described by a Poisson distribution. However, the second important aspect of their model is that each soluble precursor can initiate a new cellulose chain. Thus, because an enzyme or cell is continually producing precursors throughout the synthesis duration, both the number of polymers per enzyme and the number per cell increases when a precursor initiates a new chain. Under these hypothetical conditions, Eq. (30) would then demonstrate a positive exponent, meaning that β was greater than α . But, since the opposite was found experimentally, that α was greater than β , it must be further concluded that a polymeric precursor is not capable of randomly introducing a new cellulose chain. Therefore, if a polymeric precursor exists, its incorporation into cellulose chains or microfibrils cannot be referred to as a simple polymeric-association process, but rather as a process where much greater control of the destinies of the precursors is exercised.

Although the experimental results are in contradiction to the proposed mechanism of Colvin and Leppard (7), no such contradiction exists for the proposed mechanism of Brown et al. (15) and Zaar (16). The high relative rates of polymer elongation are consistent with the concept of an enzyme continually connected to its cellulose chain, because the entire production of each enzyme would then serve to elongate only one single cellulose chain, rather than being distributed over several chains. Furthermore, the fact that the rate constant α was determined to be greater than β is compatible with their observation that a microfibrillar ribbon does not reach its maximum width until after cellular replication, producing a time delay in the introduction of new isolatable cellulose molecules that would result in an effective decrease in their numbers per cell with time. From Table II, it is apparent that

this time-delay may be relatively short as demonstrated by experiments No. 7 and 10 where α is approximately equal to β , or relatively long as demonstrated by experiment No. 9 where α is much greater than β .

CONCLUSIONS

This study has demonstrated that the production of bacterial cellulose exhibits approximate first-order kinetics for the increase in the number of polymers, in the mass of the polymers, and in the relative length of the polymers measured by the second moment of the number distribution, for several generations. It has also demonstrated that the number of polymers per cell effectively decreases for the entire culture during synthesis.

Contrary to what has previously been proposed, however, these results are not compatible with a Poisson polymerization mechanism (4). The experimental polydispersities, measured from the GPC chromatograms of the bacterial cellulose samples, exhibit values significantly above 2, while the predicted Poisson polydispersities, on the other hand, will never exceed this value. The experimental polydispersities are much greater than the predicted theoretical values because the second distribution moment is much larger than what would be theoretically predicted.

Furthermore, because the number of polymers per cell decreases rather than increases, the results are in contradiction with the mechanism proposed by Colvin and Leppard (7). In this mechanism, soluble polymeric precursors randomly associate together either to form new cellulose molecules or to elongate previously established molecules. The results, however, are not in contradiction with the mechanism proposed by Brown et al. (15) and Zaar (16) that each cellulose chain remains attached to its synthesizing enzyme. Apparently, the bacterial cell rigorously controls both the introduction of new polymers and their subsequent polymerization.

NOMENCLATURE

A	Avogadro's number
D_2	The slope of a GPC calibration curve
$\overline{DP}_n(t)$	The number-average DP measured after a synthesis duration of t
$\overline{DP}_n(\gamma)$	The number-average DP measured after a synthesis duration of γ
$\overline{DP}_n(\lambda, \gamma)$	The theoretical, Poisson-predicted number-average DP
$\overline{DP}_w(t)$	The weight-average DP measured after a synthesis duration of t
$\overline{DP}_w(\gamma)$	The weight-average DP measured after a synthesis duration of γ
$\overline{DP}_w(\lambda, \gamma)$	The theoretical, Poisson-predicted weight-average DP
k	The number of stochastic events
k_{as}	The weight-fraction of all alkali-soluble material to the total mass
k_i	The weight-fraction of the isolated cellulose to the total cellulose
k_{ic}	The weight-fraction of the isolated cellulose to the total mass
k_s	The weight-fraction of the alkali-soluble cellulose to the total cellulose
K	The ratio of the number of polymers to the number of cells
K'	The empirical coefficient for the exponential increase in the experimentally determined number-average degree of polymerization
M	The total mass of a bacterial culture after a duration of synthesis
M_k	The molecular weight of a cellulose polymer with k number of stochastic polymerization events
M_0	The initial mass of a bacterial culture upon inoculation at time $t = 0$
\overline{M}_n	The number-average molecular weight
n	The total number of cellulose polymers
$n_i(t)$	The number of cellulose polymers isolated after an alkali extraction and a synthesis duration of t
$n_i(\gamma)$	The number of cellulose polymers isolated after an alkali extraction and a synthesis duration of γ

n_0	The initial number of polymers inoculated into a new medium
$n(k, \lambda, \gamma)$	The total number of cellulose polymers predicted by a Poisson distribution, a number distribution
N	The total number of bacterial cells
N_0	The initial number of bacterial cells inoculated into a new medium
$p[k, \lambda, (\gamma - \gamma')]$	The Poisson polymerization function
P	A GPC dispersion correction factor determined experimentally
P_t	The true GPC correction factor for dispersion
$u_j(\lambda, \gamma)$	The moments of a Poisson polymerization distribution
$v_j(\gamma)$	The moments of an experimentally determined polymerization distribution
w	The total mass of a bacterial culture after a duration of synthesis
$w_{ic}(t)$	The total mass of cellulose isolated after an alkaline extraction and a synthesis duration of t
$w_{ic}(\gamma)$	The total mass of cellulose isolated after an alkaline extraction and a synthesis duration of γ
$w(\lambda, \gamma)$	The total mass predicted by a Poisson polymerization function
w_0	The total initial mass of a bacterial culture after inoculation
x	The molecular weight of anhydroglucose, equal to 162 daltons
z	The degree of polymerization of the cellulose precursor
α	The first-order rate constant for the increase in the total number of bacterial cells, the total mass of the bacterial culture, or the total mass of isolatable cellulose
α'	The empirical first-order rate constant for the increase in the number-average molecular weight
β	The first-order rate constant for the increase in the total number of cellulose polymers extracted by alkali
δ	$\ln 2$
γ	A dimensionless time parameter determined from the ratio of real time, t , to the average time necessary for the total number of isolatable cellulose polymers to double
γ'	The time at which a particular group of polymers was introduced into the bulk of isolatable cellulose

λ	The Poisson event rate
π	Polydispersity, $\overline{DP_w}/\overline{DP_n}$
π_e	The equilibrium polydispersity, the polydispersity achieved as a limit in a Poisson polymerization given a constant value of λ
σ_v	The empirical width or standard deviation of a GPC chromatogram
τ_{cell}	The average time necessary for the number of bacterial cells to double during the logarithmic growth phase of a bacterial culture
τ_{poly}	The average time necessary for the number of cellulose polymers to double during the logarithmic growth phase of a bacterial culture

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LITERATURE CITED

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APPENDIX I

CALIBRATION NOMENCLATURE

a_x	The Mark-Houwink exponent for polymer "x" determined from the double logarithmic plot of intrinsic viscosity and molecular weight
$D_j(x)$	The coefficients of a generalized GPC polynomial calibration equation, $j = 1, 2, 3$, etc.
$G(v-\hat{v})$	The gaussian-shaped GPC chromatogram of a monodisperse polymer
$H(v)$	The GPC chromatogram for any monodisperse or polydisperse polymer
K_x	The Mark-Houwink coefficient for polymer "x" determined from the double logarithmic plot of intrinsic viscosity and molecular weight
$M(x)$	The molecular weight for a monodisperse polymer of type "x"
$\tilde{M}(x)$	The median value of a molecular weight distribution for a polymer of type "x"
$\overline{M}_n(x)$	The number-average molecular weight for a polydisperse polymer of type "x"
$\overline{M}_w(x)$	The weight-average molecular weight for a polydisperse polymer of type "x"
$\overline{M}_n'(x)$	The number-average molecular weight determined by GPC having both positional and dispersional errors
$\overline{M}_w'(x)$	The weight-average molecular weight determined by GPC having both positional and dispersional errors
$\overline{M}_{na}(x)$	The number-average molecular weight determined by GPC having only a dispersional error
$\overline{M}_{wa}(x)$	The weight-average molecular weight determined by GPC having only a dispersional error
$\overline{M}_w[t]$ or $\overline{M}_n[t]$	The weight- or number-average molecular weight determined from a GPC chromatogram utilizing a dispersion compensated calibration equation
$\overline{M}_w[T]$	The weight-average molecular weight determined from a universal calibration equation with compound positional and dispersional errors for two different types of polymers
$\overline{M}_w[\bar{V}]$	The weight-average molecular weight determined for a GPC chromatogram with a calibration equation derived from the plot of the weight-average molecular weight <u>vs.</u> the mean elution volume of a series of calibration standards
$\overline{M}_w[\tilde{V}]$	The same as above but from a plot of $\overline{M}_w[vs]$ <u>vs.</u> the median elution volume

$\overline{M}_w[\hat{V}]$	The same as above but from a plot of $\overline{M}_w[vs]$ <u>vs.</u> the model elution volume
$\overline{M}_w[vs]$	The vendor-supplied weight-average molecular weight
$P(x)$	The dispersion correction factor for a polymer of type "x"
sk	The skewing parameter for a GPC chromatogram
$u_2(x)$	The variance of the chromatogram for a monodisperse species polymer
v	The empirically measured elution volume on a GPC chromatogram
\hat{v}	The elution volume corresponding to a modal position of a monodisperse species polymer chromatogram
\overline{V}	The mean elution volume of a GPC chromatogram
\tilde{V}	The median elution volume of a GPC chromatogram
\hat{V}	The modal elution volume of a GPC chromatogram
$W(\hat{v})$	A polymer's molecular weight distribution, with the molecular weight variable transformed to the median elution volume by a median-positional calibration equation
x	A symbol denoting the type of polymer, e.g., the type of monomer it is composed of
α	The intercept of the Coll-Prusinowski formalism
β	The slope of the Coll-Prusinowski formalism
$\sigma(x)$	The standard deviation of the molecular weight distribution of a polymer of type "x"
σ_v	The standard deviation of a GPC chromatogram, determined from $(\sum (H(v) (v - \overline{V})^2) / \sum H(v))^{1/2}$
mean	Of GPC chromatogram: $(\sum (H(v) v) / \sum H(v))$ Of a lognormal molecular-weight distribution: $\ln \tilde{M}(x) + \frac{1}{2} D_2^2(x) \sigma^2(x)$
median	Of GPC chromatogram: The value of the elution volume, v , corresponding to the vertical line which divides a chromatogram into two parts having equal areas. Of a lognormal molecular-weight distribution: The value that divides the distribution into equal areas, symbolized as $\ln \tilde{M}(x)$
mode	Of GPC chromatogram: The maximum peak height Of a lognormal molecular-weight distribution: $\ln \tilde{M}(x) - D_2^2(x) \sigma^2(x)$

APPENDIX II

DISPERSION COMPENSATED CALIBRATION FOR GEL PERMEATION CHROMATOGRAPHY: THEORY AND UTILIZATION FOR DIRECT AND UNIVERSAL CALIBRATION

INTRODUCTION

The conventional method of calibrating a GPC instrument by plotting the weight-average molecular weights of a series of polymer standards against the modal or maximum peak-height positions of their respective chromatograms, results in a calibration equation that does not correctly recalculate the weight-average molecular weights of the original chromatograms used in the calibration. This is a consequence of two sources of error: instrumental spreading which produces an apparent increase in the polydispersities of the true molecular-weight distributions, and the misassignment of a weight-average molecular weight to the modal position of a chromatogram which should correspond to an intermediate value between the weight-average and the number-average molecular weights.

To circumvent this problem, McCrackin (27) has recently developed a computerized calibration technique capable of calculating a calibration equation that is a function of the true modal-positions of Gaussian monodisperse polymer chromatograms and the appropriate dispersion correction term, specific to the type of polymer. This is accomplished by determining the most probable position for a weight-average or a number-average molecular weight for a polydisperse sample on its respective chromatogram, and performing a regression analysis with similar values in a series of digitalized, input-data chromatograms of varying molecular weight. The equation determined by this technique is dispersion compensated because it can be used directly to analyze a chromatogram that has not been corrected for instrumental spreading, to obtain an accurate molecular-weight average of the type that was used in the calibration.

The purpose of this study is to demonstrate the superiority of McCrackin's technique over the common graphical technical technique, which utilizes an independently obtained dispersion correction, by documenting the nature of the positional errors inherent in the graphical technique. It is also the purpose of this study to present a valid, dispersionally correct universal calibration derived from the dispersion compensated equations. The theory of universal calibration based on infinite resolution is invalid because it neglects instrumental spreading and is subject to the propagation of serious positional errors.

THEORETICAL DEVELOPMENT

DIRECT CALIBRATION

The utilization of gel permeation chromatography to determine the molecular weight averages of polymer samples requires calibrating an instrument in regard to each molecular-weight's expected column-residency time, and determining the instrumental spreading coefficient (34). Residency time is customarily measured in elution-volume units by virtue of a constant elution flow rate. The relationship between molecular weight and elution volume is usually approximated by a linear, semilogarithmic function:

$$\ln \tilde{M}(x) = \ln M(x) = D_1(x) - D_2(x) v, \quad (34)$$

where $M(x)$ is the molecular weight of a monodisperse species-polymer of type "x," v is the elution volume, and the coefficients $D_1(x)$ and $D_2(x)$ are constants characteristic of a particular combination of instrument and polymer type. Equation (34) is the modal-positional calibration equation for a GPC instrument. It specifies the precise elution volume at which the maximum peak-height of a monodisperse polymer's chromatogram occurs. The GPC chromatograms for this species-polymer will be approximated by a Gaussian distribution (35),

$$G(v-\hat{v}) = \exp(-\frac{1}{2}(v-\hat{v})^2 / u_2(x)) / (2\pi u_2(x))^{1/2},$$

where \hat{v} is the elution volume at which the modal position occurred, and $u_2(x)$ is the variance of the Gaussian distribution. The parameter $u_2(x)$ is the spreading coefficient of the instrument and has a magnitude that is also dependent on the instrument and polymer-type (36).

By changing the variable of a Gaussian chromatogram from elution volume, v , to molecular weight via Eq. (34), a lognormal molecular weight distribution is obtained (52). But the modal position of a Gaussian distribution corresponds to the median position of a lognormal distribution ($M(x) = \tilde{M}(x)$) (38). Hence, once a series of monodisperse polymers has been eluted to determine Eq. (34), the chromatograms of the monodisperse species can be analyzed to determine the apparent molecular-weight averages. Therefore, with

$$H(v) = G(v-\hat{v}),$$

$$\overline{M}_{wa}(x) = \int_0^{\infty} H(v) \exp(D_1(x) - D_2(x) v) dv, \quad (35)$$

and

$$\overline{M}_{na}(x) = 1 \int_0^{\infty} H(v) \exp(-D_1(x) + D_2(x) v) dv, \quad (36)$$

the dispersion parameter $u_2(x)$ and the monodisperse molecular-weight $M(x)$ will be related to the apparent values through:

$$\overline{M}_{wa}(x) = M(x) \exp(\frac{1}{2} D_2^2(x) u_2(x)), \quad (37)$$

and

$$\overline{M}_{na}(x) = M(x) \exp(-\frac{1}{2} D_2^2(x) u_2(x)). \quad (38)$$

If a new dispersion parameter is defined as

$$P(x) = \exp\left(-\frac{1}{2} D_2^2(x) u_2(x)\right). \quad (39)$$

then from either Eq. (37) or Eq. (38) the dispersion parameter can be measured directly as

$$P(x) = M(x)/\overline{M}_{wa}(x) = \overline{M}_{na}(x)/M(x).$$

A polydisperse chromatogram is described as the sum of several monodisperse species chromatograms:

$$H(v) = \int_0^{\infty} G(v-\hat{v}) W(\hat{v}) d\hat{v},$$

where $W(\hat{v})$ is the weight-fraction of each separate species, or the molecular weight distribution. If the shapes of the monodisperse species-chromatograms are fairly Gaussian and the calibration equation is linear, then regardless of the actual $W(\hat{v})$, the apparent molecular-weight averages, determined for a polydisperse sample through Eq. (35) and (36), will also be convertible to the actual molecular-weight values by the same instrumental spreading coefficient of Eq. (39) (28). Thus, for polydisperse samples:

$$\overline{M}_w(x) = \overline{M}_{wa}(x) P(x), \quad (40)$$

and

$$\overline{M}_n(x) = \overline{M}_{na}(x) P^{-1}(x). \quad (41)$$

These equations, however, rely on an accurate determination of the modal-positional calibration equation, which predicts the chromatographic position of $\tilde{M}(x)$. When real, polydisperse calibration standards are utilized, only the weight-average or the number-average or both molecular weights are known, but not the median value $\tilde{M}(x)$. Generally, very narrow calibration samples are chosen where $\overline{M}_w(x) \approx \overline{M}_n(x)$ so that the error between either values and the median values would be very small. But still, an error will be propagated if either $\overline{M}_w(x)$ or $\overline{M}_n(x)$ is substituted for $\tilde{M}(x)$.

The magnitude of this error can be estimated by considering lognormal $W(\hat{v})$ functions. For such polymers, ideal infinitely resolved GPC chromatograms, where $u_2(x) = 0$, would be true depictions of $W(\hat{v})$ and would directly yield

$$\overline{M}_w(x) = \tilde{M}(x) \exp\left(\frac{1}{2} D_2^2(x) \sigma^2(x)\right), \quad (42)$$

and

$$\overline{M}_n(x) = \tilde{M}(x) \exp\left(-\frac{1}{2} D_2^2(x) \sigma^2(x)\right), \quad (43)$$

where $\sigma^2(x)$ is the actual variance of the distribution. Thus, as a result

$$\tilde{M}(x) = (\overline{M}_w(x) \overline{M}_n(x))^{1/2},$$

and

$$\overline{M}_w(x)/\overline{M}_n(x) = \exp(D_2^2(x) \sigma^2(x)). \quad (44)$$

But, when $u_2(x) > 0$, these chromatograms would still be Gaussian due to the reproductive properties of Gaussian distributions (37,38), although demonstrating empirical variance $\alpha_v^2(x) > \sigma^2(x)$. Therefore,

$$\overline{M}_{wa}(x) = \tilde{M}(x) \exp\left(\frac{1}{2} D_2^2(x) \alpha_v^2(x)\right), \quad (45)$$

and

$$\overline{M}_{na}(x) = \tilde{M}(x) \exp\left(-\frac{1}{2} D_2^2(x) \alpha_v^2(x)\right).$$

The notable feature is that $\tilde{M}(x)$ would remain constant such that

$$\tilde{M}(x) = (\overline{M}_{wa}(x) \overline{M}_{na}(x))^{1/2} = (\overline{M}_w(x) \overline{M}_n(x))^{1/2}.$$

Assigning a true molecular-weight average to a modal position will result in a positional error whose magnitude involves the actual variance of the molecular-weight distributions. By substituting Eq. (34) into both Eq. (42) and Eq. (43), the calibration equations determined from a positional error are:

$$\ln \overline{M}_w'(x) = D_1(x) - D_2(x) v + \frac{1}{2} D_2^2(x) \sigma^2(x), \quad (46)$$

and

$$\ln \overline{M}_n'(x) = D_1(x) - D_2(x) v - \frac{1}{2} D_2^2(x) \sigma^2(x), \quad (47)$$

where $\overline{M}_w'(x)$ and $\overline{M}_n'(x)$ are the molecular-weight averages uncorrected for both dispersional and positional errors. Both Eq. (46) and Eq. (47) will be referred to as positional-error calibration equations.

A direct conclusion from either Eq. (46) or Eq. (47) is that narrow, fractionated polymers will minimize the positional error because $\sigma^2(x) \approx 0$. Broader calibration standards, whose maximum chromatogram peaks are less likely to be determined precisely, will yield proportionately greater errors as $\sigma^2(x)$ increases. But, $\sigma^2(x)$ may also randomly vary within the actual calibration standard series, introducing an error that could possibly exhibit a discernible functionality with elution volume.

To avoid this positional error, McCrackin's computation technique analyzes each calibrating polymer's chromatogram for its shape and determines a calibration equation that will directly recalculate its true weight-average or number-average molecular weight (27). The form of these new calibration equations are determined by substituting Eq. (35) and Eq. (36) into Eq. (40) and Eq. (41), respectively, yielding:

$$\overline{M}_w(x) = \int_0^{\infty} H(v) \exp(D_1(x) - D_2(x) v + \ln P(x)) dv,$$

and

$$\overline{M}_n(x) = 1 / \int_0^{\infty} H(v) \exp(-D_1(x) + D_2(x) v + \ln P(x)) dv.$$

The resulting calibration equations are:

$$\ln \overline{M}_w(x) = D_1(x) - D_2(x) v + \ln P(x), \quad (48)$$

and

$$\ln \overline{M}_n(x) = D_1(x) - D_2(x) v - \ln P(x), \quad (49)$$

which will be referred to as dispersion-compensated calibration (DDC) equations. As is evidenced, DCC equations are the result of a symmetric dispersion operation about the modal-positional calibration equation.

The advantage of McCrackin's calibration technique is that a calibration equation will always be positionally and dispersionally correct regardless of whether the actual calibration-polymer distributions were broad or narrow. Furthermore, it also represents a greatly simplified method procedurally, because no independent dispersion determination is necessary and positional accuracy is assured even when only one type of molecular-weight average is known, since Eq. (48) and Eq. (49) are calculated independently of one another.

UNIVERSAL CALIBRATION

The technique of universal calibration, where the calibration equation for one type of polymer is translated into a calibration equation for another type of polymer, is based on the theory that GPC separates according to the solvated hydrodynamic volume of polymers rather than their molecular weights (39). Hydrodynamic volume is defined as the product of the intrinsic viscosity and the molecular weight of a monodisperse polymer, $[\eta]_x M(x)$, or simply in terms of molecular weight (through the Mark-Houwink constants K_x and a_x) as $K_x M^{1+a_x}(x)$. The prediction for GPC is that two polymers of different molecular weights but identical hydrodynamic volumes will elute at the same elution volume. Ideally then, the modal-positional equation for one polymer should be convertible to the second polymer by manipulation of the Mark-Houwink constants. But this is theoretically only valid for infinite resolution (39). As demonstrated previously [Eq. (37) and Eq. (38)], the point of elution for a monodisperse polymer is defined as the modal position of the monodisperse species-chromatogram. Therefore, the possibility that two monodisperse polymers

with equal mean hydrodynamic volumes, but dissimilar spreading coefficients, may have different median values for their hydrodynamic volume distributions and thus different modal values for their respective chromatograms is not considered. Furthermore, the reality of polydispersity and its potential for producing a positional-error is also ignored by this theory.

Significantly, the empirical basis for a universal calibration has been the repeated observation (39-42) that the product of the weight-average molecular weight and the intrinsic viscosity for a series of polydisperse polymers will be colinear in respect to modal elution-volumes with the same product for a second series of polydisperse polymers of differing Mark-Houwink constants,

$$[\eta]_1 \overline{M}_w(1) = [\eta]_2 \overline{M}_w(2). \quad (50)$$

Equation (50), however, has been interpreted as only an approximation to reality where the weight-averages are actually very close in value to the number-averages. But what is important is that each elution-volume increment of polymer solution after separation will be isolated as a polydisperse solution due to noninfinite resolution. Therefore, each elution-volume increment will have an intrinsic viscosity which measures a number-average hydrodynamic volume (24,43) and a viscosity-average molecular weight. Because the viscosity-average molecular weight is frequently approximated by the weight-average molecular weight, Eq. (50) actually predicts that the weight-average of each elution-volume increment for one polymer type will be proportional to the weight-average for a second polymer type,

$$K_1 \overline{M}_w^{1+a_1}(1) = K_2 \overline{M}_w^{1+a_2}(2). \quad (51)$$

In effect, a weight-average DCC equation describes the weight-average molecular weight that each elution-volume increment will have for a polymer solution that has been separated with a particular dispersion coefficient of $P(x)$. This is the reason

that a GPC chromatogram can be analyzed directly with such an equation to yield an accurate weight-average for the entire polymer or polymer solution. Thus, Eq. (51) at first inspection seems capable of interconverting one weight-average DCC equation directly into another. But the specific dispersion coefficient that one polymer-type may have is independent of the other. Therefore, although dispersion coefficients are not translatable, the equality of Eq. (51) still remains, allowing the modal-positional equation of polymer "1" to be described in terms of the modal-positional equation of polymer "2" and both dispersion coefficients. Thus, by substituting Eq. (48) for both polymers into Eq. (51):

$$\ln \tilde{M}(1) = \alpha + \beta \ln \tilde{M}(2) - \beta \ln P(2) + \ln P(1), \quad (52)$$

where $\alpha = \ln (K_2/K_1)/(1 + a_1)$,

$$\beta = (1 + a_2)/(1 + a_1).$$

What is notable about Eq. (52) is that when $\ln P(1) = \ln P(2) = 0$, the standard calibration-equation transformation utilized for universal calibration, known as the Coll-Prusinowski formalism (44,45), is obtained. Thus, when a true modal-positional calibration equation for polymer "2" is translated via the Coll-Prusinowski formalism, both dispersion coefficients of Eq. (52) are neglected. This neglect would be inconsequential if instrumental resolution of both polymers was infinite, or if $\beta \ln P(2) = \ln P(1)$. But since the former condition is impossible and the latter, at best, serendipitous, at least a dispersional error will always be expected.

Analogously, the application of the Coll-Prusinowski formalism to a positional-error equation [Eq. (46)], derived from the assignment of weight-average molecular weights to the modal positions of the calibration chromatograms, results in included errors that also rely on fortuitous relationships between the positional error and the dispersional error for minimization.

Thus to avoid both dispersional errors and positional errors, it would be advantageous to be able to translate a DCC equation for polymer "2" directly into a DCC equation for polymer "1." Therefore, applying the principle of dispersion compensation to the modal-positional Eq. (52) yields:

$$\ln \bar{M}_w(1) = \alpha + \beta \ln \tilde{M}(2) - \beta \ln P(2) + 2 \ln P(1), \quad (53)$$

and

$$\ln \bar{M}_n(1) = \alpha + \beta \ln \tilde{M}(2) - \beta \ln P(2). \quad (54)$$

The elimination of the dispersion coefficient $P(1)$ in Eq. (54) is noteworthy because this parameter could not be derived without independent dispersion measurements. When both DDC equations for polymer "2" [Eq. (48) and Eq. (49)] are translated directly by the Coll-Prusinowski formalism the results are:

$$\ln \bar{M}_w(1) = \alpha + \beta \ln \tilde{M}(2) + \beta \ln P(2), \quad (55)$$

and

$$\ln \bar{M}_n(1) = \alpha + \beta \ln \tilde{M}(2) - \beta \ln P(2). \quad (56)$$

The comparison of Eq. (54) and Eq. (56) demonstrates that the number-average DCC equation is a true universal form and can be translated into the number-average DCC equation for any other polymer whose Mark-Houwink constants are known, through the Coll-Prusinowski formalism. The comparison of Eq. (53) and Eq. (55) demonstrates that the weight-average DDC equation will not translate through the Coll-Prusinowski formalism. When one such weight-average equation is translated, a dispersion error will result between what is obtained and the true relationship.

METHODS

Styragel columns (Waters Associates) with the nominal exclusion limits of 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 nm were utilized with purified tetrahydrofuran (THF) as the

elution solvent. The flow rate was 2 mL/min. A Perkin-Elmer LC-55B spectrophotometer was operated at 235 nm for the cellulose tricarbanilates and 225 nm for the polystyrene calibration standards. The polystyrene standards were obtained from the manufacturers listed in Table V. Cellulose tricarbanilates were prepared and injected into the GPC columns according to the methods of Schroeder and Haigh (46).

All calculations were performed by computer programs obtained from reference No. 27. Two programs are available: Program Calib calibrates GPC columns by the method described in reference No. 27, and program GPC computes the molecular weight averages from chromatograms of polymer samples.

RESULTS AND DISCUSSION

DIRECT CALIBRATION

The chromatograms of ten polystyrene samples which were utilized as calibration standards are depicted in Fig. 10. Each chromatogram has been normalized to the same area, and has been characterized by the calculation of its statistical parameters: in particular its mean \bar{V} , median \tilde{V} , mode \hat{V} , and the standard deviation α_v with respect to elution volume. These values for each chromatogram are listed in Table V along with their respective vendor-supplied, weight-average molecular weights, $\bar{M}_w[vs]$.

The modal value, \hat{V} , is the parameter customarily selected for graphical calibration of GPC instruments because it corresponds to the easily identifiable maximum-peak-height of a chromatogram. Both \bar{V} and \tilde{V} as well as the modal value, \hat{V} , will be equal when the chromatograms are symmetrical, but all three parameters will not be equal when skewing is present. The degree of skewing for each chromatogram is measured by

$$sk = (\bar{V} - \hat{V}) / \alpha_v,$$

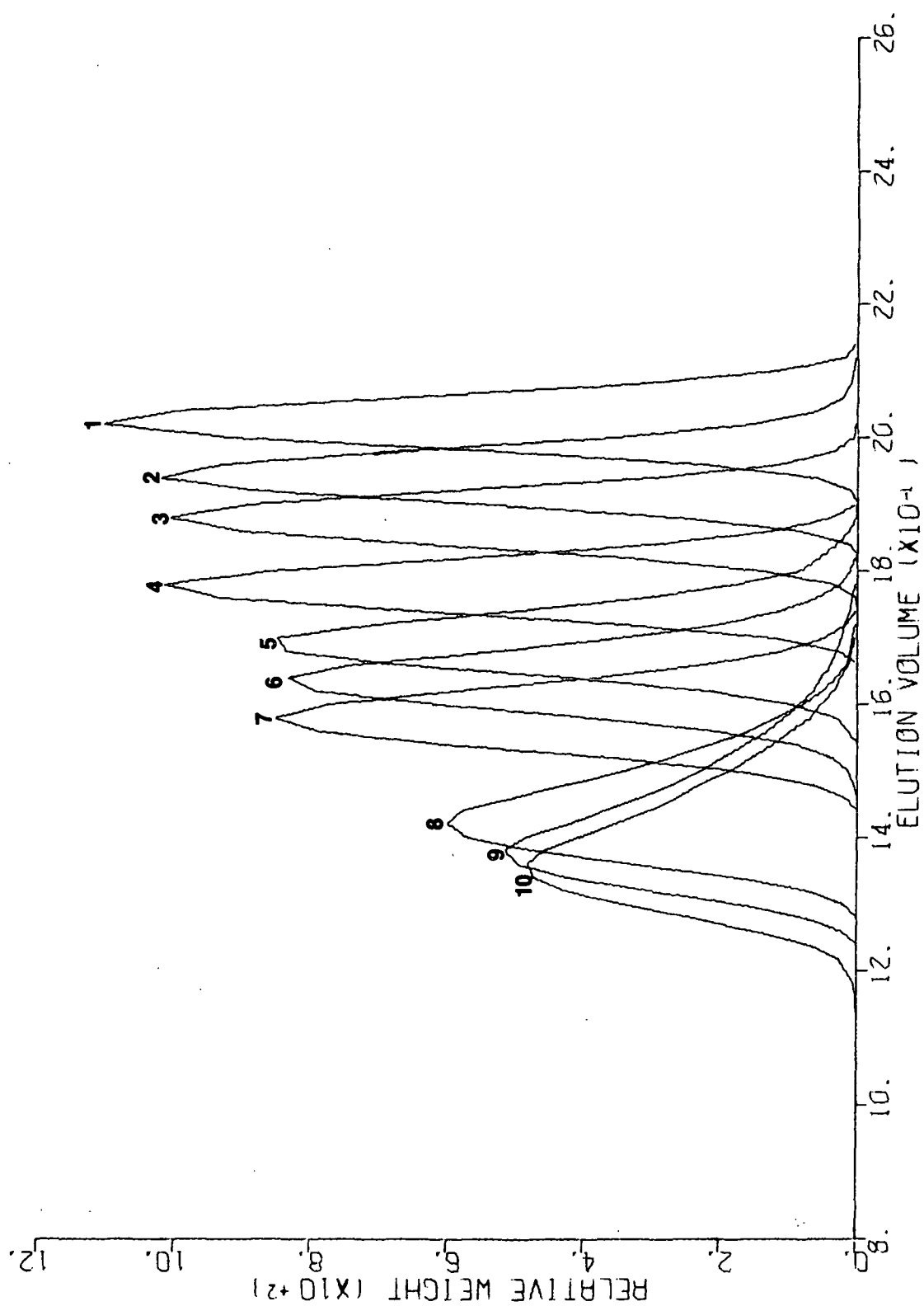


Figure 10. The GPC chromatograms of ten polystyrene calibration standards.

and, as can be seen from Table V, chromatograms No. 1 through 7 are symmetric since $\bar{V} = \tilde{V} = \hat{V}$ and $sk = 0$; however, for chromatograms No. 8, 9, and 10, $\bar{V} > \tilde{V} > \hat{V}$ and $sk > 0$. They are obviously non-Gaussian. Specifically, this means that for the skewed chromatograms, the modal elution volume will not correspond positionally to $\tilde{M}(x)$. Thus, a modified positional-error equation will result from their inclusion in a graphical calibration. The extent of this modification can be estimated by assuming that $\tilde{M}(x)$ may possibly reside closer to either \bar{V} or \tilde{V} , and that the positional-error equation determined from the symmetric chromatograms is valid through the skewed chromatograms.

TABLE V

VENDOR-SUPPLIED WEIGHT-AVERAGE MOLECULAR WEIGHTS
AND GPC STATISTICAL CHROMATOGRAPHIC PARAMETERS

Samp.	Supp.	$\bar{M}_w[vs]$	\bar{V}	\tilde{V}	\hat{V}	α_v	sk
1	PCC	4.80×10^3	202	202	202	3.49	0.0
2	PCC	1.03×10^3	194	194	194	3.88	0.0
3	PCC	1.98×10^4	188	188	188	3.85	0.0
4	PCC	5.10×10^4	178	178	178	3.81	0.0
5	DPR	1.25×10^5	170	170	170	4.85	0.0
6	PIB	2.39×10^5	164	164	164	4.95	0.0
7	WA	4.70×10^5	158	158	158	4.62	0.0
8	PCC	1.80×10^6	145	144	142	6.98	0.430
9	WA	2.70×10^6	143	141	138	9.13	0.548
10	PIB	3.56×10^6	139	138	136	8.82	0.340

PCC = Pressure Chemical Company.

DPR = Dow Physical Research Lab.

PIB = Polytechnic Institute of Brooklyn.

WA = Waters Associates.

For all ten polystyrene chromatograms, three calibration equations were derived from a linear regression of the logarithm of the vendor-supplied weight-average molecular weight and the elution volume parameters. The three calibration equations obtained were:

$$\begin{aligned}\ln \bar{M}_w[\bar{V}] &= 29.87 - 0.1064 \bar{V}, \\ \ln \bar{M}_w[\tilde{V}] &= 29.42 - 0.1040 \tilde{V},\end{aligned}\tag{57}$$

and

$$\ln \bar{M}_w[\hat{V}] = 28.67 - 0.09987 \hat{V}.$$

But the single calibration equation that was determined from the symmetric chromatograms No. 1 through 7 was

$$\ln \bar{M}_w[\tilde{V}] = 29.45 - 0.1041 \tilde{V}\tag{58}$$

Since Eq. (58) is approximately equal to Eq. (57), the median elution volume \tilde{V} is assumed to be positionally correct for $\tilde{M}(x)$. Thus, the observable effect of utilizing either \bar{V} or \hat{V} for the skewed chromatograms instead of \tilde{V} is that linear error functions are included that affect both the slope and intercept of Eq. (57).

The dispersion-compensated calibration equation obtained via McCrackin's computer program for all ten chromatograms was

$$\ln \bar{M}_w[t] = 28.93 - 0.1017 v.\tag{59}$$

It varies in both slope and intercept from Eq. (57). If Eq. (57) was a true modal-positional calibration equation, the expectation is that only the intercepts would differ due to the inclusion of the constant dispersional parameter $\ln P(x)$ in Eq. (59). However, due to the positional error of assigning the weight-average molecular weight to a modal position, the actual form of Eq. (57) is given by Eq. (46). But by substituting Eq. (39), (42), and (45) into Eq. (40) and simplifying, the

standard relationship between dispersion and distribution variances for the symmetrical chromatograms was obtained (47):

$$\sigma^2(x) = \sigma_v^2(x) - u_2(x). \quad (60)$$

By further substitution of Eq. (60) into Eq. (46), the positional-error equation can be compared to the DCC equation,

$$\ln \overline{M}_w'(x) = \ln \overline{M}_w(x) + \frac{1}{2} D_2^2(x) \sigma_v^2. \quad (61)$$

Thus, the fact that the parameter σ_v actually does show a functionality with elution volume in Table V explains why the slope of Eq. (57) was also different from the slope of Eq. (59). But, more important, Eq. (61) demonstrates that any weight-average molecular weight calculated from a positional-error equation will always be greater than the weight-average calculated from a true modal-positional equation with dispersion correction performed either independently or simultaneously, as in McCrackin's method. This is true because no distribution variance $\sigma^2(x)$ can be obtained for any polymer standards that will yield an empirical σ_v^2 equal to zero. Therefore, $\overline{M}_w'(x)$ is a weight-average molecular weight that is uncorrected for dispersion and positional errors, and will still yield incorrect values when corrected for just peak dispersion.

Table VI compares the vendor-supplied weight-average molecular weights with the weight-averages calculated for each polystyrene chromatogram by all four calibration equations mentioned, in a numerical approximation of Eq. (35). As expected, the dispersion-compensated calibration equation [Eq. (59)] demonstrated calculated weight-average values $\overline{M}_w[t]$ with an insignificant average-deviation from the vendor-supplied values. In contrast, all three graphical methods of calibration produced larger average-deviations. The values calculated from the median calibration [Eq. (57)] $\overline{M}_w[\tilde{V}]$ were consistently larger than the values calculated from McCrackin's

TABLE VI
CALCULATED WEIGHT AVERAGES

Samp.	$\overline{M}_w[vs]$	$\overline{M}_w[t]$	$\Delta\%$ ^a	$\overline{M}_w[\overline{V}]$	$\Delta\%$ ^a	$\overline{M}_w[\overline{V}]$	$\Delta\%$ ^a	$M_w[\hat{V}]$	$\Delta\%$ ^a
1	4.80×10^3	4.54×10^3	-5.4	4.55×10^3	-5.2	4.67×10^3	-2.7	5.04×10^3	5.0
2	1.03×10^4	1.02×10^4	-1.0	1.06×10^4	2.9	1.07×10^4	3.9	1.12×10^4	8.7
3	1.98×10^4	2.00×10^4	1.0	2.15×10^4	8.6	2.13×10^4	7.6	2.16×10^4	9.1
4	5.10×10^4	5.51×10^4	8.0	6.20×10^4	21.6	6.00×10^4	17.6	5.85×10^4	14.7
5	1.25×10^5	1.32×10^5	5.6	1.55×10^5	24.0	1.47×10^5	17.6	1.38×10^5	10.4
6	2.39×10^5	2.38×10^5	-0.4	2.88×10^5	20.5	2.69×10^5	12.6	2.46×10^5	2.9
7	4.70×10^5	4.18×10^5	-11.1	5.17×10^5	10.0	4.77×10^5	1.5	4.28×10^5	-8.9
8	1.80×10^6	1.84×10^6	2.2	2.45×10^6	36.1	2.18×10^6	21.1	1.83×10^6	1.7
9	2.70×10^6	2.55×10^6	-5.6	3.46×10^6	28.1	1.05×10^6	13.0	2.52×10^6	-6.7
10	3.56×10^6	3.65×10^6	2.5	5.04×10^6	41.6	4.40×10^6	23.6	3.59×10^6	0.8
		$\overline{\Delta}$	-0.4		18.8		11.6		3.8
		$ \overline{\Delta\%} $	4.3		19.9		12.1		6.9

^aRelative error based on $\overline{M}_w[vs]$ values.

calibration $\overline{M}_w[t]$, as predicted by Eq. (61). The effect of positional discrepancy between the modal elution-volume \hat{V} and the value of $\tilde{M}(x)$ from the skewed chromatograms is depicted by the $\overline{M}_w[\hat{V}]$ values for chromatograms No. 8, 9, and 10. When skewing is positive ($sk > 0$) the modal value reduces the effect of positional error and produces values very close to both the dispersion compensated and the vendor-supplied.

The substitution of Eq. (60) into Eq. (44) results in an expression that may be used to calculate the number-average molecular weight of each calibrating-polymer's chromatogram,

$$\overline{M}_n(x) = \overline{M}_w(x) \exp(-D_2^2(x) \alpha_v^2)/P^2(x).$$

Knowledge of the spreading coefficient $P(x)$, however, is required. But McCrackin's technique can also be used in an iterative fashion to determine which value of $P(x)$ will yield a number-average DDC equation that is parallel to the previously determined weight-average DCC equation. For various values of $P(x)$, Table VII demonstrates the response of the computer program in calculating the new number-average DCC equations. Since McCrackin's technique is not restricted to linear equations, quadratic coefficients will occur when included-error functions are also nonlinear. As can be seen in Table VII, the best value of $P(x)$ which gave the lowest relative error, and a number-average DCC equation approximately parallel to the weight-average DCC equation was $P(x) = 0.97$.

In this calculation, $\overline{M}_w[t]$ values should be used for $\overline{M}_w(x)$ instead of $M_w[vs]$ because $\overline{M}_n[t]$ values are needed to complement the respective weight-averages. Therefore, the experimental errors associated with $\overline{M}_w[vs]$ values do not enter into these calculations.

TABLE VII

NUMBER AVERAGE DCC EQUATIONS

P	D ₁ (2)	D ₂ (2)	D ₃ (2)	RE ^a
0.99	29.00	-0.1022	1.185 x 10 ⁻⁶	0.61
0.98	29.02	-0.1021	1.154 x 10 ⁻⁶	0.47
0.97	29.01	-0.1018	0.0	0.42
0.96	29.03	-0.1018	4.240 x 10 ⁻⁹	0.59
0.95	29.09	-0.1023	1.458 x 10 ⁻⁶	0.46

^aRelative error determined by McCrackin's computer program by comparing calculated number-average values against inputted number-average values.

Utilizing Eq. (58) to analyze the chromatogram of polystyrene SRM-706 (48), a $\overline{M}_w[t] = 2.80 \times 10^5$ was calculated. This value fell between the weight-average values reported for light scattering (2.58×10^5) and sedimentation equilibrium (2.88×10^5). The number-average molecular weight calculated at $P(x) = 0.97$ gave $\overline{M}_n[t] = 1.49 \times 10^5$, which compared to the membrane osmometry number-average of 1.37×10^5 . The calculated GPC polydispersity, ($\overline{M}_w[t]/\overline{M}_n[t] = 1.88$) was identical to the polydispersity calculated from the ratio of the light scattering to the membrane osmometry values; however, both the calculated weight-average and number-average molecular weights have a relative error of 8% greater than the direct measurement. This error is within acceptable limits for GPC.

UNIVERSAL CALIBRATION

With the intention of obtaining DCC equations for cellulose tricarbanilate (CTC), both the weight-average and the number-average DCC equations for polystyrene were translated via the Coll-Prusinski formalism. The constants α and β were calculated using the respective Mark-Houwink constants [$K_2 = 1.179 \times 10^{-2}$ and $a_2 = 0.74$ for polystyrene, $K_1 = 2.010 \times 10^{-3}$ and $a_1 = 0.92$ for CTC (40)]. In this

situation, however, it was found necessary to subtract the Ptitsyn-Eisner correction factor (0.15) from α to account for CTC's deviations from random coiling (44,49). The discrepancy that was discovered involved the calculation of a number-average greater than the vendor-supplied weight-average for the narrowest sample (N-5) when the correction was not included.

The resulting calibration equations were:

$$\ln \overline{M}_w(1) = 26.99 - 0.09217 v, \quad (62)$$

and

$$\ln \overline{M}_n(1) = 27.06 - 0.09226 v. \quad (63)$$

As expected and demonstrated in Table VIII, the weight-average values $\overline{M}_w[T]$ calculated for the CTC chromatograms via Eq. (62) differed from the vendor-supplied weight-average values $\overline{M}_w[vs]$. By virtue of the difference between Eq. (53) and Eq. (55), the dispersional error would be:

$$P^2(1)/P^2(2) = \overline{M}_w[vs]/\overline{M}_w[T].$$

From Table VIII and with $P(2) = 0.97$, the dispersional coefficient was found to have an average value of $P(1) = 0.67$. This smaller $P(x)$ value for CTC is consistent with a greater degree of dispersion due to a greater rigidity of the polymers in solution (49). The correct DCC equation for the weight-average values was determined to be:

$$\ln \overline{M}_w(1) = 26.26 - 0.09217 v, \quad (64)$$

which yielded much more reasonable values for $\overline{M}_w[t] \approx \overline{M}_w[vs]$. The dispersion coefficient calculated from Eq. (63) and Eq. (64) was $P(1) = 0.67 = \exp\left(\frac{1}{2}(26.26 - 27.06)\right)$.

By simple inspection of Eq. (57) in regard to Eq. (59), the application of the Coll-Prusinowski formalism to the positional-error equation for the polystyrenes would have resulted in weight-average molecular weights that were even larger than

the $\overline{M}_w[T]$ values. This is a consequence of the incorporation of both dispersional errors and positional errors. This fact can be demonstrated by simple application of the Coll-Prusinowski formalism to Eq. (61):

$$\ln \overline{M}_w(1) = \alpha + \beta \ln \tilde{M}(2) + \beta \ln P(2) + \frac{1}{2} \beta D_2^2(2) \alpha_v^2,$$

which is Eq. (55) with a positional error term that will always be greater than zero because α_v^2 will never be zero.

TABLE VIII
CELLULOSE TRICARBANILATE DATA

Samp. ^a	$\overline{DP}_w[vs]^b$	$\overline{DP}_w[T]$	P(1) ^c	$\overline{DP}_w[t]$	$\overline{DP}_n[t]$
N-5	550	1130	0.68	542	473
N-30	1000	2310	0.64	1110	816
N-70	1300	2480	0.70	1270	764
		avg.	0.67		

^aSamples were obtained from Hercules, Inc.

^b $\overline{DP} = M/519$.

^cCalculated from $P^2(1) = P^2 \beta(2) \overline{DP}_w[vs]/\overline{DP}_w[T]$.

SUMMARY

The calculation of reliably accurate molecular-weight averages for polymer samples through gel permeation chromatography depends on the determination of a true modal-positional calibration equation and an instrumental spreading coefficient. The common graphical procedure of assigning a weight-average molecular weight value to a modal position on a chromatogram will result, however, in an equation that has a positional error that will produce calculated weight-averages always greater in value than the dispersionally correct value. Because the dispersion correction will generally not be appropriate for the positional error, further correction of these values for dispersional error will not properly correct these values. To avoid this

situation, McCrackin has developed a computerized calibration technique which will calculate a dispersion compensated calibration equation for either weight-average or number-average molecular weight that is dispersionally and positionally correct. Dispersion compensated calibration equations can be used to directly analyze a GPC chromatogram to obtain an accurate value for the particular molecular weight-average originally used to obtain the equation without further dispersion correction.

The problems of positional and dispersional errors are generally magnified when a calibration equation for one polymer type is converted, by the principle of universal calibration, into the equation for another polymer type whose identical hydrodynamic volume corresponds to a different molecular weight. But because a number-average dispersion-compensated calibration equation is properly balanced for the correct dispersion terms, it is a true universal form and can be translated into the number-average dispersion-compensated equation for another polymer type through a universal calibration transformation.

APPENDIX III

BACTERIAL CELLULOSE CHROMATOGRAMS

The following is a listing of the digitalized chromatograms utilized in this study of bacterial cellulose kinetics. The chromatograms were digitalized every 2-mL increment. For each sample there are two duplicates. The tabular columns are labeled as:

v The elution volume at which the height of the chromatogram was read.

H(v) The height of the chromatogram above its baseline.

I(v) The integral value of the chromatogram at v.

All chromatograms were analyzed with McCrackin's program GPC which uses Simpson's numerical integration technique. The DCC equations, Eq. (63) and Eq.(64), were used to calculate $\overline{M}_w[t]$ and $\overline{M}_n[t]$ values for the bacterial cellulose tricarbanilate samples. For each duplicate the actual values for the molecular weight averages are given as \overline{DP}_s ($\overline{DP} = \overline{M}/519$) directly above the tabulations as:

$$\overline{DP}_w[t]/\overline{DP}_n[t].$$

2464/1290

[illegible]

SAMPLE 7-2

2687/1298

V	H(V)	I(V)
1.0400000E-00	0.0000000E-00	1.0000000E-00
1.0600000E-00	0.0000000E-00	1.999301E-01
1.0800000E-00	0.0000000E-00	9.988225E-01
1.1000000E-00	0.0000000E-00	9.953843E-01
1.1200000E-00	0.0000000E-00	9.886146E-01
1.1400000E-00	0.0000000E-00	9.777726E-01
1.1600000E-00	0.0000000E-00	9.621199E-01
1.1800000E-00	0.0000000E-00	9.410455E-01
1.2000000E-00	0.0000000E-00	9.146802E-01
1.2200000E-00	0.0000000E-00	8.833929E-01
1.2400000E-00	0.0000000E-00	8.475152E-01
1.2600000E-00	0.0000000E-00	8.074431E-01
1.2800000E-00	0.0000000E-00	7.644737E-01
1.3000000E-00	0.0000000E-00	7.193378E-01
1.3200000E-00	0.0000000E-00	6.725517E-01
1.3400000E-00	0.0000000E-00	6.243510E-01
1.3600000E-00	0.0000000E-00	5.753211E-01
1.3800000E-00	0.0000000E-00	5.253067E-01
1.4000000E-00	0.0000000E-00	4.756407E-01
1.4200000E-00	0.0000000E-00	4.267915E-01
1.4400000E-00	0.0000000E-00	3.798122E-01
1.4600000E-00	0.0000000E-00	3.355902E-01
1.4800000E-00	0.0000000E-00	2.945228E-01
1.5000000E-00	0.0000000E-00	2.576238E-01
1.5200000E-00	0.0000000E-00	2.250380E-01
1.5400000E-00	0.0000000E-00	1.973642E-01
1.5600000E-00	0.0000000E-00	1.739285E-01
1.5800000E-00	0.0000000E-00	1.527928E-01
1.6000000E-00	0.0000000E-00	1.337795E-01
1.6200000E-00	0.0000000E-00	1.168227E-01
1.6400000E-00	0.0000000E-00	1.038369E-01
1.6600000E-00	0.0000000E-00	9.192442E-02
1.6800000E-00	0.0000000E-00	8.150734E-02
1.7000000E-00	0.0000000E-00	7.248083E-02
1.7200000E-00	0.0000000E-00	6.485496E-02
1.7400000E-00	0.0000000E-00	5.87641E-02
1.7600000E-00	0.0000000E-00	5.399215E-02
1.7800000E-00	0.0000000E-00	4.959215E-02
1.8000000E-00	0.0000000E-00	4.559215E-02
1.8200000E-00	0.0000000E-00	4.195496E-02
1.8400000E-00	0.0000000E-00	3.87641E-02
1.8600000E-00	0.0000000E-00	3.599215E-02
1.8800000E-00	0.0000000E-00	3.359215E-02
1.9000000E-00	0.0000000E-00	3.159215E-02
1.9200000E-00	0.0000000E-00	2.995496E-02
1.9400000E-00	0.0000000E-00	2.87641E-02
1.9600000E-00	0.0000000E-00	2.799215E-02
1.9800000E-00	0.0000000E-00	2.759215E-02
2.0000000E-00	0.0000000E-00	2.739215E-02

2776/1249

V	H(V)	I(V)
1.0400000E-01	0.0000000E-01	1.0000000E-00
1.0600000E-01	0.0000000E-00	1.999615E-01
1.0800000E-01	0.0000000E-00	9.994293E-01
1.1000000E-01	0.0000000E-00	9.976004E-01
1.1200000E-01	0.0000000E-00	9.933429E-01
1.1400000E-01	0.0000000E-00	9.857097E-01
1.1600000E-01	0.0000000E-00	9.740631E-01
1.1800000E-01	0.0000000E-00	9.577008E-01
1.2000000E-01	0.0000000E-00	9.361315E-01
1.2200000E-01	0.0000000E-00	9.091799E-01
1.2400000E-01	0.0000000E-00	8.771251E-01
1.2600000E-01	0.0000000E-00	8.404137E-01
1.2800000E-01	0.0000000E-00	7.99611E-01
1.3000000E-01	0.0000000E-00	7.570258E-01
1.3200000E-01	0.0000000E-00	7.125705E-01
1.3400000E-01	0.0000000E-00	6.670132E-01
1.3600000E-01	0.0000000E-00	6.245436E-01
1.3800000E-01	0.0000000E-00	5.847191E-01
1.4000000E-01	0.0000000E-00	5.475906E-01
1.4200000E-01	0.0000000E-00	5.127015E-01
1.4400000E-01	0.0000000E-00	4.803044E-01
1.4600000E-01	0.0000000E-00	4.502573E-01
1.4800000E-01	0.0000000E-00	4.223720E-01
1.5000000E-01	0.0000000E-00	3.961222E-01
1.5200000E-01	0.0000000E-00	3.719297E-01
1.5400000E-01	0.0000000E-00	3.495432E-01
1.5600000E-01	0.0000000E-00	3.287027E-01
1.5800000E-01	0.0000000E-00	3.092365E-01
1.6000000E-01	0.0000000E-00	2.916198E-01
1.6200000E-01	0.0000000E-00	2.758364E-01
1.6400000E-01	0.0000000E-00	2.619148E-01
1.6600000E-01	0.0000000E-00	2.495775E-01
1.6800000E-01	0.0000000E-00	2.387364E-01
1.7000000E-01	0.0000000E-00	2.293105E-01
1.7200000E-01	0.0000000E-00	2.213057E-01
1.7400000E-01	0.0000000E-00	2.147333E-01
1.7600000E-01	0.0000000E-00	2.093607E-01
1.7800000E-01	0.0000000E-00	2.051907E-01
1.8000000E-01	0.0000000E-00	2.02007E-01

2776/1249

$I(\nu)$	ν
1	000000E 00
9	998828E -01
9	9985930E -01
9	9948251E -01
9	9762205E -01
9	9762205E -01
9	9606134E -01
9	9405637E -01
9	9405636E -01
9	960079E -01
8	8634401E -01
8	857751E -01
7	7744861E -01
7	744861E -01
6	3033218E -01
6	343216E -01
6	376735E -01
5	9122061E -01
5	457125E -01
4	585400E -01
4	159893E -01
3	734964E -01
3	309806E -01
2	885945E -01
2	471679E -01
2	77650E -01
1	711664E -01
1	381046E -01
1	091593E -01
8	442903E -02
6	365609E -02
4	671669E -02
4	311753E -02
2	252746E -02
1	471412E -02
9	284854E -03
5	663693E -03
3	274202E -03
1	820564E -04
4	491444E -04
1	084805E -04
0	0

v	$H(v)$	$I(v)$	v	$H(v)$	$I(v)$
0	0.700000	0.000000	0	0.000000	0.000000
1	0.389999	0.993340	1	0.000000	0.998007
2	0.539999	0.982198	2	0.000000	0.976382
3	0.168000	0.797194	3	0.000000	0.911536
4	0.196000	0.600306	4	0.000000	0.781855
5	0.125000	0.332587	5	0.000000	0.306755
6	0.260999	0.994487	6	0.000000	0.968747
7	0.180999	0.612154	7	0.000000	0.575374
8	0.480999	0.715929	8	0.000000	0.137438
9	0.615999	0.722133	9	0.000000	0.661047
10	0.682998	0.721337	10	0.000000	0.161930
11	0.637998	0.710833	11	0.000000	0.646225
12	0.637998	0.714516	12	0.000000	0.120452
13	0.503000	0.307458	13	0.000000	0.605192
14	0.295000	0.707498	14	0.000000	0.153413
15	0.495000	0.904621	15	0.000000	0.742666
16	0.335000	0.214417	16	0.000000	0.374610
17	0.189000	0.299309	17	0.000000	0.442781
18	0.140000	0.594236	18	0.000000	0.732064
19	0.069000	0.204890	19	0.000000	0.137916
20	0.034000	0.725723	20	0.000000	0.857936
21	0.018000	0.462765	21	0.000000	0.320922
22	0.009000	0.202936	22	0.000000	0.726180
23	0.004000	0.110504	23	0.000000	0.842263
24	0.002000	0.452213	24	0.000000	0.222209
25	0.001000	0.878046	25	0.000000	0.723220
26	0.000500	0.045831	26	0.000000	0.122091
27	0.000200	0.878337	27	0.000000	0.847773
28	0.000100	0.493265	28	0.000000	0.991773
29	0.000050	0.621585	29	0.000000	0.325596
30	0.000020	0.818512	30	0.000000	0.722446
31	0.000010	0.621849	31	0.000000	0.385675
32	0.000005	0.135041	32	0.000000	0.930675
33	0.000002	0.364469	33	0.000000	0.613227
34	0.000001	0.935056	34	0.000000	0.282799
35	0.000000	0.000000	35	0.000000	0.428625
36	0.000000	0.000000	36	0.000000	0.951966
37	0.000000	0.000000	37	0.000000	0.351966
38	0.000000	0.000000	38	0.000000	0.000000
39	0.000000	0.000000	39	0.000000	0.000000
40	0.000000	0.000000	40	0.000000	0.000000
41	0.000000	0.000000	41	0.000000	0.000000
42	0.000000	0.000000	42	0.000000	0.000000
43	0.000000	0.000000	43	0.000000	0.000000
44	0.000000	0.000000	44	0.000000	0.000000
45	0.000000	0.000000	45	0.000000	0.000000
46	0.000000	0.000000	46	0.000000	0.000000
47	0.000000	0.000000	47	0.000000	0.000000
48	0.000000	0.000000	48	0.000000	0.000000
49	0.000000	0.000000	49	0.000000	0.000000
50	0.000000	0.000000	50	0.000000	0.000000
51	0.000000	0.000000	51	0.000000	0.000000
52	0.000000	0.000000	52	0.000000	0.000000
53	0.000000	0.000000	53	0.000000	0.000000
54	0.000000	0.000000	54	0.000000	0.000000
55	0.000000	0.000000	55	0.000000	0.000000
56	0.000000	0.000000	56	0.000000	0.000000
57	0.000000	0.000000	57	0.000000	0.000000
58	0.000000	0.000000	58	0.000000	0.000000
59	0.000000	0.000000	59	0.000000	0.000000
60	0.000000	0.000000	60	0.000000	0.000000
61	0.000000	0.000000	61	0.000000	0.000000
62	0.000000	0.000000	62	0.000000	0.000000
63	0.000000	0.000000	63	0.000000	0.000000
64	0.000000	0.000000	64	0.000000	0.000000
65	0.000000	0.000000	65	0.000000	0.000000
66	0.000000	0.000000	66	0.000000	0.000000
67	0.000000	0.000000	67	0.000000	0.000000
68	0.000000	0.000000	68	0.000000	0.000000
69	0.000000	0.000000	69	0.000000	0.000000
70	0.000000	0.000000	70	0.000000	0.000000
71	0.000000	0.000000	71	0.000000	0.000000
72	0.000000	0.000000	72	0.000000	0.000000
73	0.000000	0.000000	73	0.000000	0.000000
74	0.000000	0.000000	74	0.000000	0.000000
75	0.000000	0.000000	75	0.000000	0.000000
76	0.000000	0.000000	76	0.000000	0.000000
77	0.000000	0.000000	77	0.000000	0.000000
78	0.000000	0.000000	78	0.000000	0.000000
79	0.000000	0.000000	79	0.000000	0.000000
80	0.000000	0.000000	80	0.000000	0.000000
81	0.000000	0.000000	81	0.000000	0.000000
82	0.000000	0.000000	82	0.000000	0.000000
83	0.000000	0.000000	83	0.000000	0.000000
84	0.000000	0.000000	84	0.000000	0.000000
85	0.000000	0.000000	85	0.000000	0.000000
86	0.000000	0.000000	86	0.000000	0.000000
87	0.000000	0.000000	87	0.000000	0.000000
88	0.000000	0.000000	88	0.000000	0.000000
89	0.000000	0.000000	89	0.000000	0.000000
90	0.000000	0.000000	90	0.000000	0.000000
91	0.000000	0.000000	91	0.000000	0.000000
92	0.000000	0.000000	92	0.000000	0.000000
93	0.000000	0.000000	93	0.000000	0.000000
94	0.000000	0.000000	94	0.000000	0.000000
95	0.000000	0.000000	95	0.000000	0.000000
96	0.000000	0.000000	96	0.000000	0.000000
97	0.000000	0.000000	97	0.000000	0.000000
98	0.000000	0.000000	98	0.000000	0.000000
99	0.000000	0.000000	99	0.000000	0.000000
100	0.000000	0.000000	100	0.000000	0.000000

SAMPLE 7-5

4237/1431

4742/1511

V	H(V)	I(V)	V	H(V)	I(V)
1.000000E-01	0.400000E-01	1.000000E-01	9.400000E-01	0.500000E-01	1.000000E-01
1.000000E-01	0.509999E-01	1.000000E-01	9.600000E-01	0.900000E-01	1.998941E-01
1.000000E-01	0.652000E-01	1.000000E-01	9.800000E-01	0.200000E-01	9.993726E-01
1.000000E-01	0.139000E-01	1.000000E-01	9.000000E-01	0.000000E-01	9.981472E-01
1.000000E-01	0.665000E-01	1.000000E-01	9.000000E-01	0.000000E-01	9.957483E-01
1.000000E-01	0.166500E-01	1.000000E-01	9.000000E-01	0.000000E-01	9.914092E-01
1.000000E-01	0.253000E-01	1.000000E-01	9.000000E-01	0.000000E-01	9.837932E-01
1.000000E-01	0.052998E-01	1.000000E-01	9.000000E-01	0.000000E-01	9.718428E-01
1.000000E-01	0.445999E-01	1.000000E-01	9.000000E-01	0.000000E-01	9.551767E-01
1.000000E-01	0.822998E-01	1.000000E-01	9.000000E-01	0.000000E-01	9.335830E-01
1.000000E-01	0.229998E-01	1.000000E-01	9.000000E-01	0.000000E-01	9.075325E-01
1.000000E-01	0.405000E-01	1.000000E-01	9.000000E-01	0.000000E-01	8.776045E-01
1.000000E-01	0.305000E-01	1.000000E-01	9.000000E-01	0.000000E-01	8.441083E-01
1.000000E-01	0.555000E-01	1.000000E-01	9.000000E-01	0.000000E-01	8.073405E-01
1.000000E-01	0.570599E-01	1.000000E-01	9.000000E-01	0.000000E-01	7.677095E-01
1.000000E-01	0.337000E-01	1.000000E-01	9.000000E-01	0.000000E-01	7.254118E-01
1.000000E-01	0.328999E-01	1.000000E-01	9.000000E-01	0.000000E-01	6.8071156E-01
1.000000E-01	0.335000E-01	1.000000E-01	9.000000E-01	0.000000E-01	6.342973E-01
1.000000E-01	0.682998E-01	1.000000E-01	9.000000E-01	0.000000E-01	5.870488E-01
1.000000E-01	0.412000E-01	1.000000E-01	9.000000E-01	0.000000E-01	5.395323E-01
1.000000E-01	0.277000E-01	1.000000E-01	9.000000E-01	0.000000E-01	4.928114E-01
1.000000E-01	0.339999E-01	1.000000E-01	9.000000E-01	0.000000E-01	4.484288E-01
1.000000E-01	0.223999E-01	1.000000E-01	9.000000E-01	0.000000E-01	4.073609E-01
1.000000E-01	0.213999E-01	1.000000E-01	9.000000E-01	0.000000E-01	3.701531E-01
1.000000E-01	0.221399E-01	1.000000E-01	9.000000E-01	0.000000E-01	3.357328E-01
1.000000E-01	0.987998E-01	1.000000E-01	9.000000E-01	0.000000E-01	3.022793E-01
1.000000E-01	0.476000E-01	1.000000E-01	9.000000E-01	0.000000E-01	2.691100E-01
1.000000E-01	0.157000E-01	1.000000E-01	9.000000E-01	0.000000E-01	2.360200E-01
1.000000E-01	0.183900E-01	1.000000E-01	9.000000E-01	0.000000E-01	2.0322210E-01
1.000000E-01	0.539000E-01	1.000000E-01	9.000000E-01	0.000000E-01	1.713893E-01
1.000000E-01	0.153000E-01	1.000000E-01	9.000000E-01	0.000000E-01	1.413723E-01
1.000000E-01	0.240000E-01	1.000000E-01	9.000000E-01	0.000000E-01	1.139529E-01
1.000000E-01	0.855000E-01	1.000000E-01	9.000000E-01	0.000000E-01	8.50624E-02
1.000000E-01	0.479999E-01	1.000000E-01	9.000000E-01	0.000000E-01	6.850631E-02
1.000000E-01	0.309999E-01	1.000000E-01	9.000000E-01	0.000000E-01	5.069552E-02
1.000000E-01	0.670000E-01	1.000000E-01	9.000000E-01	0.000000E-01	3.534051E-02
1.000000E-01	0.320000E-01	1.000000E-01	9.000000E-01	0.000000E-01	2.434779E-02
1.000000E-01	0.400000E-01	1.000000E-01	9.000000E-01	0.000000E-01	1.543336E-03
1.000000E-01	0.700000E-01	1.000000E-01	9.000000E-01	0.000000E-01	9.034336E-03
1.000000E-01	0.740000E-01	1.000000E-01	9.000000E-01	0.000000E-01	4.785717E-03
1.000000E-01	0.760000E-01	1.000000E-01	9.000000E-01	0.000000E-01	2.260447E-04
1.000000E-01	0.820000E-01	1.000000E-01	9.000000E-01	0.000000E-01	8.509755E-04
1.000000E-01	1.800000E-01	1.000000E-01	9.000000E-01	0.000000E-01	1.920462E-04
1.000000E-01	1.840000E-01	1.000000E-01	9.000000E-01	0.000000E-01	3.933907E-06

3241/1269

[illegible]

2166/911

[illegible]

2911/1175

[illegible]

3576/1154

3797/1250

ν	$H(\nu)$	$I(\nu)$	ν	$H(\nu)$	$I(\nu)$
0	0.1500000	1.0000000	0	0.0000000	0.0000000
1	0.9899999	0.9916532	1	0.0000000	0.0000000
2	0.4500000	0.9591522	2	0.0000000	0.0000000
3	0.1500000	0.8911282	3	0.0000000	0.0000000
4	0.0930000	0.8781793	4	0.0000000	0.0000000
5	0.3400000	0.6330770	5	0.0000000	0.0000000
6	0.6090000	0.4307707	6	0.0000000	0.0000000
7	0.8950000	0.1931076	7	0.0000000	0.0000000
8	0.1390000	0.9106800	8	0.0000000	0.0000000
9	0.3220000	0.5844388	9	0.0000000	0.0000000
10	0.4760000	0.2233786	10	0.0000000	0.0000000
11	0.7079998	0.8236425	11	0.0000000	0.0000000
12	0.6109999	0.7823598	12	0.0000000	0.0000000
13	0.7489999	0.2598888	13	0.0000000	0.0000000
14	0.7409999	0.9960295	14	0.0000000	0.0000000
15	0.6679998	0.5546955	15	0.0000000	0.0000000
16	0.5330000	0.1109418	16	0.0000000	0.0000000
17	0.4420000	0.6737488	17	0.0000000	0.0000000
18	0.3800000	0.2533846	18	0.0000000	0.0000000
19	0.4330000	0.8533840	19	0.0000000	0.0000000
20	0.4200000	0.4628840	20	0.0000000	0.0000000
21	0.3160000	0.6768048	21	0.0000000	0.0000000
22	0.4290000	0.2981344	22	0.0000000	0.0000000
23	0.4330000	0.9054646	23	0.0000000	0.0000000
24	0.3030000	0.5149078	24	0.0000000	0.0000000
25	0.2220000	0.1358019	25	0.0000000	0.0000000
26	0.1913000	0.7794292	26	0.0000000	0.0000000
27	0.6433000	0.4533514	27	0.0000000	0.0000000
28	0.2200000	0.1632504	28	0.0000000	0.0000000
29	0.1733000	0.1325040	29	0.0000000	0.0000000
30	0.2000000	0.0841040	30	0.0000000	0.0000000
31	0.8520000	0.2551839	31	0.0000000	0.0000000
32	0.4799999	0.7954289	32	0.0000000	0.0000000
33	0.3059999	0.6402602	33	0.0000000	0.0000000
34	0.7999999	0.8036622	34	0.0000000	0.0000000
35	0.7000000	0.2006290	35	0.0000000	0.0000000
36	0.4799999	0.7872590	36	0.0000000	0.0000000
37	0.1200000	0.0125725	37	0.0000000	0.0000000
38	0.7999999	0.1080254	38	0.0000000	0.0000000
39	0.4000000	0.8504298	39	0.0000000	0.0000000
40	0.4000000	0.7241278	40	0.0000000	0.0000000
41	0.7000000	0.4758933	41	0.0000000	0.0000000
42	0.9999999	0.5555328	42	0.0000000	0.0000000
43	0.0000000	0.0000000	43	0.0000000	0.0000000
44	0.0000000	0.0000000	44	0.0000000	0.0000000
45	0.0000000	0.0000000	45	0.0000000	0.0000000

[illegible]

3764/1288

[illegible]

4335/1467

[illegible]

5576/1712

$$H(\nu)$$
$$(A)I$$

A

$$H(\Delta)$$
$$I(v)$$

9.	800000E	01
1.	000000E	02
1.	020000E	02
1.	040000E	02
1.	060000E	02
1.	080000E	02
1.	100000E	02
1.	120000E	02
1.	140000E	02
1.	160000E	02
1.	180000E	02
1.	200000E	02
1.	220000E	02
1.	240000E	02
1.	260000E	02
1.	280000E	02
1.	300000E	02
1.	320000E	02
1.	340000E	02
1.	360000E	02
1.	380000E	02
1.	400000E	02
1.	420000E	02
1.	440000E	02
1.	460000E	02
1.	480000E	02
1.	500000E	02
1.	520000E	02
1.	540000E	02
1.	560000E	02
1.	580000E	02
1.	600000E	02
1.	620000E	02
1.	640000E	02
1.	660000E	02
1.	680000E	02
1.	700000E	02
1.	720000E	02
1.	740000E	02
1.	760000E	02
1.	780000E	02
1.	800000E	02
1.	820000E	02
1.	840000E	02

[illegible]

1.000000E+00	1.994404E-01	9.96196E-01	9.88821E-01	9.786257E-01	9.621390E-01	9.402710E-01	9.135392E-01	8.824728E-01	8.474284E-01	8.08815E-01	7.669638E-01	7.223384E-01	6.762308E-01	6.297415E-01	5.831601E-01	5.362960E-01	4.893031E-01	4.436051E-01	4.016216E-01	3.648599E-01	3.329282E-01	3.02541E-01	2.739721E-01	2.449113E-01	2.160109E-01	1.872092E-01	1.582030E-01	1.318502E-01	1.069743E-01	8.461487E-02	6.513613E-02	4.858130E-02	3.505498E-02	2.436817E-02	1.617527E-02	1.027209E-02	6.277241E-03	3.976609E-03	2.457704E-03	1.4801154E-04	3.60178E-04	0.0
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[illegible][illegible]

1	.000000E+00
11	.000017E+00
99	.991317E-01
99	.995970E-01
99	.890590E-01
99	.770174E-01
99	.600065E-01
99	.377544E-01
99	.103392E-01
88	.786902E-01
88	.432631E-01
88	.044503E-01
77	.623159E-01
77	.172554E-01
66	.705963E-01
66	.236578E-01
55	.766883E-01
55	.295965E-01
44	.825349E-01
44	.362920E-01
33	.933126E-01
33	.558673E-01
33	.233119E-01
22	.933214E-01
22	.641392E-01
22	.352588E-01
22	.066377E-01
11	.780927E-01
11	.501253E-01
99	.233293E-01
77	.774067E-02
55	.909073E-02
44	.335630E-02
33	.046137E-02
22	.033758E-02
11	.273280E-02
77	.448018E-03
11	.918733E-03
77	.621646E-04
55	.591610E-04
00	.000000E+00

v	$H(v)$	$I(v)$	v	$H(v)$	$I(v)$
1	0.7000000	0.0000000	9	1.0000000	1.0000000
2	0.8333333	0.0000000	10	1.0000000	1.0000000
3	0.6666667	0.0000000	11	1.0000000	1.0000000
4	0.5000000	0.0000000	12	1.0000000	1.0000000
5	0.3333333	0.0000000	13	1.0000000	1.0000000
6	0.1666667	0.0000000	14	1.0000000	1.0000000
7	0.0000000	0.0000000	15	1.0000000	1.0000000
8	0.0000000	0.0000000	16	1.0000000	1.0000000
9	0.0000000	0.0000000	17	1.0000000	1.0000000
10	0.0000000	0.0000000	18	1.0000000	1.0000000
11	0.0000000	0.0000000	19	1.0000000	1.0000000
12	0.0000000	0.0000000	20	1.0000000	1.0000000
13	0.0000000	0.0000000	21	1.0000000	1.0000000
14	0.0000000	0.0000000	22	1.0000000	1.0000000
15	0.0000000	0.0000000	23	1.0000000	1.0000000
16	0.0000000	0.0000000	24	1.0000000	1.0000000
17	0.0000000	0.0000000	25	1.0000000	1.0000000
18	0.0000000	0.0000000	26	1.0000000	1.0000000
19	0.0000000	0.0000000	27	1.0000000	1.0000000
20	0.0000000	0.0000000	28	1.0000000	1.0000000
21	0.0000000	0.0000000	29	1.0000000	1.0000000
22	0.0000000	0.0000000	30	1.0000000	1.0000000
23	0.0000000	0.0000000	31	1.0000000	1.0000000
24	0.0000000	0.0000000	32	1.0000000	1.0000000
25	0.0000000	0.0000000	33	1.0000000	1.0000000
26	0.0000000	0.0000000	34	1.0000000	1.0000000
27	0.0000000	0.0000000	35	1.0000000	1.0000000
28	0.0000000	0.0000000	36	1.0000000	1.0000000
29	0.0000000	0.0000000	37	1.0000000	1.0000000
30	0.0000000	0.0000000	38	1.0000000	1.0000000
31	0.0000000	0.0000000	39	1.0000000	1.0000000
32	0.0000000	0.0000000	40	1.0000000	1.0000000
33	0.0000000	0.0000000	41	1.0000000	1.0000000
34	0.0000000	0.0000000	42	1.0000000	1.0000000
35	0.0000000	0.0000000	43	1.0000000	1.0000000
36	0.0000000	0.0000000	44	1.0000000	1.0000000
37	0.0000000	0.0000000	45	1.0000000	1.0000000
38	0.0000000	0.0000000	46	1.0000000	1.0000000
39	0.0000000	0.0000000	47	1.0000000	1.0000000
40	0.0000000	0.0000000	48	1.0000000	1.0000000
41	0.0000000	0.0000000	49	1.0000000	1.0000000
42	0.0000000	0.0000000	50	1.0000000	1.0000000
43	0.0000000	0.0000000	51	1.0000000	1.0000000
44	0.0000000	0.0000000	52	1.0000000	1.0000000
45	0.0000000	0.0000000	53	1.0000000	1.0000000
46	0.0000000	0.0000000	54	1.0000000	1.0000000
47	0.0000000	0.0000000	55	1.0000000	1.0000000
48	0.0000000	0.0000000	56	1.0000000	1.0000000
49	0.0000000	0.0000000	57	1.0000000	1.0000000
50	0.0000000	0.0000000	58	1.0000000	1.0000000
51	0.0000000	0.0000000	59	1.0000000	1.0000000
52	0.0000000	0.0000000	60	1.0000000	1.0000000
53	0.0000000	0.0000000	61	1.0000000	1.0000000
54	0.0000000	0.0000000	62	1.0000000	1.0000000
55	0.0000000	0.0000000	63	1.0000000	1.0000000
56	0.0000000	0.0000000	64	1.0000000	1.0000000
57	0.0000000	0.0000000	65	1.0000000	1.0000000
58	0.0000000	0.0000000	66	1.0000000	1.0000000
59	0.0000000	0.0000000	67	1.0000000	1.0000000
60	0.0000000	0.0000000	68	1.0000000	1.0000000
61	0.0000000	0.0000000	69	1.0000000	1.0000000
62	0.0000000	0.0000000	70	1.0000000	1.0000000
63	0.0000000	0.0000000	71	1.0000000	1.0000000
64	0.0000000	0.0000000	72	1.0000000	1.0000000
65	0.0000000	0.0000000	73	1.0000000	1.0000000
66	0.0000000	0.0000000	74	1.0000000	1.0000000
67	0.0000000	0.0000000	75	1.0000000	1.0000000
68	0.0000000	0.0000000	76	1.0000000	1.0000000
69	0.0000000	0.0000000	77	1.0000000	1.0000000
70	0.0000000	0.0000000	78	1.0000000	1.0000000
71	0.0000000	0.0000000	79	1.0000000	1.0000000
72	0.0000000	0.0000000	80	1.0000000	1.0000000
73	0.0000000	0.0000000	81	1.0000000	1.0000000
74	0.0000000	0.0000000	82	1.0000000	1.0000000
75	0.0000000	0.0000000	83	1.0000000	1.0000000
76	0.0000000	0.0000000	84	1.0000000	1.0000000
77	0.0000000	0.0000000	85	1.0000000	1.0000000
78	0.0000000	0.0000000	86	1.0000000	1.0000000
79	0.0000000	0.0000000	87	1.0000000	1.0000000
80	0.0000000	0.0000000	88	1.0000000	1.0000000
81	0.0000000	0.0000000	89	1.0000000	1.0000000
82	0.0000000	0.0000000	90	1.0000000	1.0000000
83	0.0000000	0.0000000	91	1.0000000	1.0000000
84	0.0000000	0.0000000	92	1.0000000	1.0000000
85	0.0000000	0.0000000	93	1.0000000	1.0000000
86	0.0000000	0.0000000	94	1.0000000	1.0000000
87	0.0000000	0.0000000	95	1.0000000	1.0000000
88	0.0000000	0.0000000	96	1.0000000	1.0000000
89	0.0000000	0.0000000	97	1.0000000	1.0000000
90	0.0000000	0.0000000	98	1.0000000	1.0000000
91	0.0000000	0.0000000	99	1.0000000	1.0000000
92	0.0000000	0.0000000	100	1.0000000	1.0000000

5725/1681

[illegible]

3442/7774

[illegible]

5354/1706

ν	$H(\nu)$	$I(\nu)$	ν	$H(\nu)$	$I(\nu)$
1	0.000000	1.997627	1	0.000000	1.997627
2	0.000000	9.976493	2	0.000000	9.976493
3	0.000000	9.916647	3	0.000000	9.916647
4	0.000000	9.848926	4	0.000000	9.848926
5	0.000000	9.764350	5	0.000000	9.764350
6	0.000000	9.671729	6	0.000000	9.671729
7	0.000000	9.571094	7	0.000000	9.571094
8	0.000000	9.462983	8	0.000000	9.462983
9	0.000000	9.347597	9	0.000000	9.347597
10	0.000000	9.225677	10	0.000000	9.225677
11	0.000000	9.100831	11	0.000000	9.100831
12	0.000000	8.974575	12	0.000000	8.974575
13	0.000000	8.846277	13	0.000000	8.846277
14	0.000000	8.716406	14	0.000000	8.716406
15	0.000000	8.584452	15	0.000000	8.584452
16	0.000000	8.450617	16	0.000000	8.450617
17	0.000000	8.315390	17	0.000000	8.315390
18	0.000000	8.179183	18	0.000000	8.179183
19	0.000000	8.042474	19	0.000000	8.042474
20	0.000000	7.905746	20	0.000000	7.905746
21	0.000000	7.768330	21	0.000000	7.768330
22	0.000000	7.630799	22	0.000000	7.630799
23	0.000000	7.492746	23	0.000000	7.492746
24	0.000000	7.354574	24	0.000000	7.354574
25	0.000000	7.216466	25	0.000000	7.216466
26	0.000000	7.077833	26	0.000000	7.077833
27	0.000000	6.939183	27	0.000000	6.939183
28	0.000000	6.800545	28	0.000000	6.800545
29	0.000000	6.661907	29	0.000000	6.661907
30	0.000000	6.523274	30	0.000000	6.523274
31	0.000000	6.384638	31	0.000000	6.384638
32	0.000000	6.245999	32	0.000000	6.245999
33	0.000000	6.107365	33	0.000000	6.107365
34	0.000000	5.968729	34	0.000000	5.968729
35	0.000000	5.830094	35	0.000000	5.830094
36	0.000000	5.691457	36	0.000000	5.691457
37	0.000000	5.552819	37	0.000000	5.552819
38	0.000000	5.414183	38	0.000000	5.414183
39	0.000000	5.275545	39	0.000000	5.275545
40	0.000000	5.136907	40	0.000000	5.136907
41	0.000000	5.000000	41	0.000000	5.000000
42	0.000000	4.863094	42	0.000000	4.863094
43	0.000000	4.726183	43	0.000000	4.726183
44	0.000000	4.589274	44	0.000000	4.589274
45	0.000000	4.452365	45	0.000000	4.452365
46	0.000000	4.315457	46	0.000000	4.315457
47	0.000000	4.178545	47	0.000000	4.178545
48	0.000000	4.041638	48	0.000000	4.041638
49	0.000000	3.904729	49	0.000000	3.904729
50	0.000000	3.767820	50	0.000000	3.767820
51	0.000000	3.630911	51	0.000000	3.630911
52	0.000000	3.494002	52	0.000000	3.494002
53	0.000000	3.357094	53	0.000000	3.357094
54	0.000000	3.220183	54	0.000000	3.220183
55	0.000000	3.083274	55	0.000000	3.083274
56	0.000000	2.946365	56	0.000000	2.946365
57	0.000000	2.809457	57	0	

v	$H(v)$	$I(v)$	v	$H(v)$	$I(v)$
1	0.35	0.00	9	0.99	0.00
2	0.32	0.00	10	0.99	0.00
3	0.28	0.00	11	0.99	0.00
4	0.25	0.00	12	0.99	0.00
5	0.22	0.00	13	0.99	0.00
6	0.20	0.00	14	0.99	0.00
7	0.18	0.00	15	0.99	0.00
8	0.17	0.00	16	0.99	0.00
9	0.16	0.00	17	0.99	0.00
10	0.15	0.00	18	0.99	0.00
11	0.14	0.00	19	0.99	0.00
12	0.13	0.00	20	0.99	0.00
13	0.12	0.00	21	0.99	0.00
14	0.11	0.00	22	0.99	0.00
15	0.10	0.00	23	0.99	0.00
16	0.09	0.00	24	0.99	0.00
17	0.08	0.00	25	0.99	0.00
18	0.07	0.00	26	0.99	0.00
19	0.06	0.00	27	0.99	0.00
20	0.05	0.00	28	0.99	0.00
21	0.04	0.00	29	0.99	0.00
22	0.03	0.00	30	0.99	0.00
23	0.02	0.00	31	0.99	0.00
24	0.01	0.00	32	0.99	0.00
25	0.00	0.00	33	0.99	0.00
26	0.00	0.00	34	0.99	0.00
27	0.00	0.00	35	0.99	0.00
28	0.00	0.00	36	0.99	0.00
29	0.00	0.00	37	0.99	0.00
30	0.00	0.00	38	0.99	0.00
31	0.00	0.00	39	0.99	0.00
32	0.00	0.00	40	0.99	0.00
33	0.00	0.00	41	0.99	0.00
34	0.00	0.00	42	0.99	0.00
35	0.00	0.00	43	0.99	0.00
36	0.00	0.00	44	0.99	0.00
37	0.00	0.00	45	0.99	0.00
38	0.00	0.00	46	0.99	0.00
39	0.00	0.00	47	0.99	0.00
40	0.00	0.00	48	0.99	0.00
41	0.00	0.00	49	0.99	0.00
42	0.00	0.00	50	0.99	0.00
43	0.00	0.00	51	0.99	0.00
44	0.00	0.00	52	0.99	0.00
45	0.00	0.00	53	0.99	0.00
46	0.00	0.00	54	0.99	0.00
47	0.00	0.00	55	0.99	0.00
48	0.00	0.00	56	0.99	0.00
49	0.00	0.00	57	0.99	0.00
50	0.00	0.00	58	0.99	0.00
51	0.00	0.00	59	0.99	0.00
52	0.00	0.00	60	0.99	0.00
53	0.00	0.00	61	0.99	0.00
54	0.00	0.00	62	0.99	0.00
55	0.00	0.00	63	0.99	0.00
56	0.00	0.00	64	0.99	0.00
57	0.00	0.00	65	0.99	0.00
58	0.00	0.00	66	0.99	0.00
59	0.00	0.00	67	0.99	0.00
60	0.00	0.00	68	0.99	0.00
61	0.00	0.00	69	0.99	0.00
62	0.00	0.00	70	0.99	0.00
63	0.00	0.00	71	0.99	0.00
64	0.00	0.00	72	0.99	0.00
65	0.00	0.00	73	0.99	0.00
66	0.00	0.00	74	0.99	0.00
67	0.00	0.00	75	0.99	0.00
68	0.00	0.00	76	0.99	0.00
69	0.00	0.00	77	0.99	0.00
70	0.00	0.00	78	0.99	0.00
71	0.00	0.00	79	0.99	0.00
72	0.00	0.00	80	0.99	0.00
73	0.00	0.00	81	0.99	0.00
74	0.00	0.00	82	0.99	0.00
75	0.00	0.00	83	0.99	0.00
76	0.00	0.00	84	0.99	0.00
77	0.00	0.00	85	0.99	0.00
78	0.00	0.00	86	0.99	0.00
79	0.00	0.00	87	0.99	0.00
80	0.00	0.00	88	0.99	0.00
81	0.00	0.00	89	0.99	0.00
82	0.00	0.00	90	0.99	0.00
83	0.00	0.00	91	0.99	0.00
84	0.00	0.00	92	0.99	0.00
85	0.00	0.00	93	0.99	0.00
86	0.00	0.00	94	0.99	0.00
87	0.00	0.00	95	0.99	0.00
88	0.00	0.00	96	0.99	0.00
89	0.00	0.00	97	0.99	0.00
90	0.00	0.00	98	0.99	0.00
91	0.00	0.00	99	0.99	0.00
92	0.00	0.00	100	0.99	0.00

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V	H(V)	I(V)	V	H(V)	I(V)
9	0.4	1	9	0.9	1
1	1.3	2	1	1.9	2
1	2.4	2	1	2.9	2
1	3.4	2	1	3.9	2
1	4.5	2	1	4.9	2
1	5.6	2	1	5.9	2
1	6.7	2	1	6.9	2
1	7.8	2	1	7.9	2
1	8.9	2	1	8.9	2
1	9.0	2	1	9.0	2
1	0.1	2	1	0.1	2
1	1.2	2	1	1.2	2
1	2.3	2	1	2.3	2
1	3.4	2	1	3.4	2
1	4.5	2	1	4.5	2
1	5.6	2	1	5.6	2
1	6.7	2	1	6.7	2
1	7.8	2	1	7.8	2
1	8.9	2	1	8.9	2
1	9.0	2	1	9.0	2
1	0.1	2	1	0.1	2
1	1.2	2	1	1.2	2
1	2.3	2	1	2.3	2
1	3.4	2	1	3.4	2
1	4.5	2	1	4.5	2
1	5.6	2	1	5.6	2
1	6.7	2	1	6.7	2
1	7.8	2	1	7.8	2
1	8.9	2	1	8.9	2
1	9.0	2	1	9.0	2
1	0.1	2	1	0.1	2
1	1.2	2	1	1.2	2
1	2.3	2	1	2.3	2
1	3.4	2	1	3.4	2
1	4.5	2	1	4.5	2
1	5.6	2	1	5.6	2
1	6.7	2	1	6.7	2
1	7.8	2	1	7.8	2
1	8.9	2	1	8.9	2
1	9.0	2	1	9.0	2
1	0.1	2	1	0.1	2
1	1.2	2	1	1.2	2
1	2.3	2	1	2.3	2
1	3.4	2	1	3.4	2
1	4.5	2	1	4.5	2
1	5.6	2	1	5.6	2
1	6.7	2	1	6.7	2
1	7.8	2	1	7.8	2
1	8.9	2	1	8.9	2
1	9.0	2	1	9.0	2
1	0.1	2	1	0.1	2
1	1.2	2	1	1.2	2
1	2.3	2	1	2.3	2
1	3.4	2	1	3.4	2
1	4.5	2	1	4.5	2
1	5.6	2	1	5.6	2
1	6.7	2	1	6.7	2
1	7.8	2	1	7.8	2
1	8.9	2	1	8.9	2
1	9.0	2	1	9.0	2
1	0.1	2	1	0.1	2
1	1.2	2	1	1.2	2
1	2.3	2	1	2.3	2
1	3.4	2	1	3.4	2
1	4.5	2	1	4.5	2
1	5.6	2	1	5.6	2
1	6.7	2	1	6.7	2
1	7.8	2	1	7.8	2
1	8.9	2	1	8.9	2
1	9.0	2	1	9.0	2
1	0.1	2	1	0.1	2
1	1.2	2	1	1.2	2
1	2.3	2	1	2.3	2
1	3.4	2	1	3.4	2
1	4.5	2	1	4.5	2
1	5.6	2	1	5.6	2
1	6.7	2	1	6.7	2
1	7.8	2	1	7.8	2
1	8.9	2	1	8.9	2
1	9.0	2	1	9.0	2
1	0.1	2	1	0.1	2
1	1.2	2	1	1.2	2
1	2.3	2	1	2.3	2
1	3.4	2	1	3.4	2
1	4.5	2	1	4.5	2
1	5.6	2	1	5.6	2
1	6.7	2	1	6.7	2
1	7.8	2	1	7.8	2
1	8.9	2	1	8.9	2
1	9.0	2	1		

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5395/1385

[illegible]